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(54) Title: CONTROL OF PROTEIN SYNTHESIS, AND SCREENING METHOD FOR AGENTS

(57) Abstract

A method for screening for agents capable of affecting the activity of kinases GSK3 and PKB is disclosed. The method involves assessing the phosphorylation of PKB on two amino acids on the PKB molecule particularly.

1   Control of protein synthesis, and screening method for
2   agents.
3
4   The present invention relates to the control of
5   glycogen metabolism and protein synthesis, in
6   particular through the use of insulin.
7
8   Many people with diabetes have normal levels of insulin
9   in their blood, but the insulin fails to stimulate
10   muscle cells and fat cells in the normal way (type II
11   diabetes). Currently it is believed that there is a
12   breakdown in the mechanism through which insulin
13   signals to the muscle and fat cells.
14
15   The enzyme glycogen synthase kinase-3 (GSK3) (Embi et
16   al., 1980) is implicated in the regulation of several
17   physiological processes, including the control of
18   glycogen (Parker et al., 1983) and protein (Welsh et
19   al., 1993) synthesis by insulin, modulation of the
20   transcription factors AP-1 and CREB (Nikolaki et al, de
21   Groot et al., 1993 and Fiol et al 1994), the
22   specification of cell fate in Drosophila (Siegfied et
23   al., 1992) and dorsoventral patterning in Xenopus
24   embryos (He et al., 1995). GSK3 is inhibited by serine

1   phosphorylation in response to insulin or growth

2   factors (Welsh et al., 1993, Hughes et al., 1994, Cross

3   et al., 1994 and Saito et al., 1994) and in $vitro$ by

4   either MAP kinase-activated protein (MAPKAP) kinase-1

5   (also known as $p90^{rsk}$) or P70 ribosomal S6 kinase ($p70^{S6k}$)

6   (Sutherland et al., 1993 and Sutherland et al., 1994).

7

8   We have now found, however, that agents which prevent

9   the activation of both MAPKAP kinase-1 and $p70^{S6k}$ by

10   insulin in $vivo$ do not block the phosphorylation and

11   inhibition of GSK3. Another insulin-stimulated protein

12   kinase inactivates GSK3 under these conditions, and we

13   demonstrate that it is the product of the proto-

14   oncogene Akt (also known as RAC or PKB; herein referred

15   to as "PKB").

16

17   GSK3 is inhibited in response to insulin with a half

18   time of two min, slightly slower than the half time for

19   activation of PKBα (one min). Inhibition of GSK3 by

20   insulin results in its phosphorylation at the same

21   serine residue (serine 21) which is targeted by PKBα in

22   vitro. Like the activation of PKBα, the inhibition of

23   GSK3 by insulin is prevented by phosphatidyl inositol

24   (PI-3) kinase inhibitors wortmannin and LY 294002. The

25   inhibition of GSK3 is likely to contribute to the

26   increase in the rate of glycogen synthesis (Cross et

27   al., 1994) and translation of certain mRNAs by insulin

28   (Welsh et al., 1994).

29

30   Two isoforms of PKB, termed PKBα (Coffer & Woodgett,

31   1991), PKBβ (Cheng et al., 1992) and PKBγ (Konishi et

32   al., 1995) have been identified and characterised.

33   PKBβ, also known as RACβ and Akt-2, is over-expressed

34   in a significant number of ovarian (Cheng et al., 1992)

35   and pancreatic (Cheng et al., 1996) cancers and is

36   over-expressed in the breast cancer epithelial cell

1   line MCF7. PKB is composed of an N-terminal pleckstrin
2   homology (PH) domain, followed by a catalytic domain
3   and a short C-terminal tail. The catalytic domain is
4   most similar to cyclic AMP-dependent protein kinase
5   (PKA, 65% similarity) and to protein kinase C (PKC, 75%
6   similarity) findings that gave rise to two of its
7   names, namely PKB (i.e. between PKA and PKC) and RAC
8   (Related to A and C kinase).
9
10   Many growth factors trigger the activation of
11   phosphatidylinositol (PI) 3-kinase, the enzyme which
12   converts PI 4,5 bisphosphate (PIP2) to the putative
13   second messenger PI 3,4,5 trisphosphate (PIP3), and PKB
14   lies downstream of PI 3-kinase (Franke et al., 1995).
15   PKBα is converted from an inactive to an active form
16   with a half time of about one minute when cells are
17   stimulated with PDGF (Franke et al., 1995), EGF or
18   basic FGF (Burgering & Coffer, 1995) or insulin (Cross
19   et al., 1995 and Kohn et al., 1995) or perpervanadate
20   (Andjelkovic et al., 1996). Activation of PKB by
21   insulin or growth factors is prevented if the cells are
22   preincubated with inhibitors of PI 3-kinase (wortmannin
23   or LY 294002) or by overexpression of a dominant
24   negative mutant of PI 3-kinase (Burgering & Coffer
25   1995). Mutation of the tyrosine residues in the PDGF
26   receptor that when phosphorylated bind to PI 3-kinase
27   also prevent the activation of PKBα (Burgering &
28   Coffer, 1995 and Franke et al., 1995).
29
30   The present invention thus provides the use of PKB, its
31   analogues, isoforms, inhibitors, activators and/or the
32   functional equivalents thereof to regulate glycogen
33   metabolism and/or protein synthesis, in particular in
34   disease states where glycogen metabolism and/or protein
35   synthesis exhibits abnormality, for example in the
36   treatment of type II diabetes; also in the treatment of

1   cancer, such as ovarian, breast and pancreatic cancer.
2   A composition comprising such agents is also covered by
3   the present invention, and the use of such a
4   composition for treatment of disease states where
5   glycogen metabolism and/or protein synthesis exhibit
6   abnormality.
7
8   The present invention also provides a novel peptide
9   comprising the amino acid sequence Arg-Xaa-Arg-Yaa-Zaa-
10   Ser/Thr-Hyd, where Xaa is any amino acid, Yaa and Zaa
11   are any amino acid (preferably not glycine), and Hyd is
12   a large hydrophobic residue such as Phe or Leu, or a
13   functional equivalent thereof. Represented in single
14   letter code, a suitable peptide would be RXRX'X'S/TF/L,
15   where X' can be any amino acid, but is preferably not
16   glycine; glycine can in fact be used, but other amino
17   acids are preferred. Typical peptides include
18   GRPRTSSFAEG, RPRAATC or functional equivalents thereof.
19   The peptide is a substrate for measuring PKB activity.
20
21   The invention also provides a method for screening for
22   substances which inhibit the activation of PKB in vivo
23   by preventing its interaction with PIP3 or PI3,4-bisP.
24
25   Thus the invention also provides a method of
26   determining the ability of a substance to affect the
27   activity or activation of PKB, the method comprising
28   exposing the substance to PKB and phosphatidyl inositol
29   polyphosphate (ie PIP3, PI3,4-bisP etc) and determining
30   the interaction between PKB and the phosphatidyl
31   inositol polyphosphate. The interaction between PKB
32   and the phosphatidyl inositol polyphosphate can
33   conveniently be measured by assessing the
34   phosphorylation state of PKB (preferably at T308 and/or
35   S473), eg by measuring transfer of radiolabelled ^{32}P
36   from the PIP3 (for example) to the PKB and/or by SDS-

1   PAGE.

3   The method of the invention can also be used for
4   identifying activators or inhibitors of GSK3, such a
5   method can comprise exposing the substance to be tested
6   to GSK3, and (optionally) a source of phosphorylation,
7   and determining the state of activation of GSK3
8   (optionally by determining the state of its
9   phosphorylation. This aspect of the invention can be
10   useful for determining the suitability of a test
11   substance for use in combatting diabetes, cancer, or
12   any disorder which involves irregularity of protein
13   synthesis or glycogen metabolism.

15   The invention also provides a method for screening for
16   inhibitors or activators of enzymes that catalyse the
17   phosphorylation of PKB, the method comprising exposing
18   the substance to be tested to
19   - one or more enzymes upstream of PKB;
20   - PKB; and (optionally)
21   - nucleoside triphosphate
22   and determining whether (and optionally to what extent)
23   the PKB has been phosphorylated on T308 and/or S473.

25   Also provided is a method of identifying agents able to
26   influence the activity of GSK3, said method comprising:

28   a.   exposing a test substance to a substrate of GSK3;

30   b.   detecting whether (and, optionally, to what
31   extent) said peptide has been phosphorylated.

33   The test substance may be an analogue, isoform,
34   inhibitor, or activator of PKB, and the above method
35   may be modified to identify those agents which
36   stimulate or inhibit PKB itself. Thus such a method

1   may comprise the following steps:

2

3   a.   exposing the test substance to a sample containing
4   PKB, to form a mixture;

5

6   b.   exposing said mixture to a peptide comprising the
7   amino acid sequence defined above or a functional
8   equivalent thereof (usually in the presence of Mg^{2+}
9   and ATP); and

10

11   c.   detecting whether (and, optionally, to what
12   extent) said peptide has been phosphorylated.

13

14   In this aspect the method of the invention can be used
15   to determine whether the substance being tested acts on
16   PKB or directly on GSK3. This can be done by comparing
17   the phosphorylation states of the peptide and PKB; if
18   the phosphorylation state of GSK3 is changed but that
19   of PKB is not then the substance being tested acts
20   directly on GSK3 without acting on PKB.
21   In a further aspect the present invention provides a
22   method of treatment of the human or non-human
23   (preferably mammalian) animal body, said method
24   comprising administering PKB, its analogues,
25   inhibitors, stimulators or functional equivalents
26   thereof to said body. Said method affects the
27   regulation of glycogen metabolism in the treated body.

28

29   The method of treatment of the present invention may be
30   of particular use in the treatment of type II diabetes
31   (where desirably an activator of PKB is used, so that
32   the down-regulation of GSK3 activity due to the action
33   of PKB is enhanced).

34

35   The method of treatment of the present invention may
36   alternatively be of particular use in the treatment of

7

cancer such as ovarian cancer (where desirably an
inhibitor of PKB is used, so that the down-regulation
of GSK3 activity due to the action of PKB is
depressed). Other cancers associated with
irregularities in the activity of PKB and/or GSK3 may
also be treated by the method, such as pancreatic
cancer, and breast cancer.

Stimulation of PKB with insulin increases activity
12-fold within 5 min and induces its phosphorylation at
Thr-308 and Ser-473. PKB transiently transfected into
cells can be activated 20-fold in response to insulin
and 46-fold in response to IGF-1 and also became
phosphorylated at Thr-308 and Ser-473. The activation
of PKB and its phosphorylation at both Thr-308 and
Ser-473 can be prevented by the phosphatidylinositol
(Pl) 3-kinase inhibitor wortmannin. The
phosphorylation of threonine 308 and serine 473 act
synergistically to activate PKB.

MAPKAP kinase-2-phosphorylated PKB at Ser-473 in vitro
increases activity seven-fold, an effect that can be
mimicked (fivefold activation) by mutating Ser-473 to
Asp. Mutation of Thr-308 to Asp also increases PKB
activity five-fold and subsequent phosphorylation of
Ser-473 by MAPKAP kinase-2 stimulates activity a
further fivefold, an effect mimicked (18-fold
activation) by mutating both Thr-308 and Ser-473 to
Asp. The activity of the Asp-308/Asp-473 double mutant
was similar to that of the fully phosphorylated enzyme
and could not be activated further by insulin. Mutation
of Thr-308 to Ala did not prevent the phosphorylation
of transfected PKB at Ser-473 after stimulation of 293
cells with insulin or IGF-1, but abolished the
activation of PKB. Similarly, mutation of Ser-473 to
Ala did not prevent the phosphorylation of transfected

1   PKB at Thr-308 but greatly reduced the activation of

2   transfected PKB. This demonstrates that the activation

3   of PKB by insulin or IGF-1 results from the

4   phosphorylation of Thr-308 and Ser-473 and that

5   phosphorylation of both residues is preferred to

6   generate a high level of PKB activity in vitro or in

7   vivo. Also, phosphorylation of Thr-308 in vivo is not

8   dependent on the phosphorylation of Ser-473 or vice

9   versa, that the phosphorylation of Thr-308 and Ser-473

10   are both dependent on PI 3-kinase activity and suggest

11   that neither Thr-308 nor Ser-473 phosphorylation is

12   catalysed by PKB itself.

13

14   Thus, it is preferred that the present invention

15   incorporates the use of any agent which affects

16   phosphorylation of PKB at amino acids 308 and/or 473,

17   for example insulin, inhibitors of PI 3-kinase such as

18   wortmannin or the like. The use of PKB, itself altered

19   at amino acids 308 and/or 473 (eg by phosphorylation

20   and/or mutation) is also suitable.

21

22   In a variation of the method of the present invention,

23   stimulation or inhibition of PKB may be assessed by

24   monitoring the phosphorylation states of amino acids

25   308 and/or 473 on PKB itself.

26

27   Different isoforms of PKB may be used or targeted in

28   the present invention; for example PKBα, β or γ.

29

30   The present invention will now be described in more

31   detail in the accompanying examples which are provided

32   by way of non-limiting illustration, and with reference

33   to the accompanying drawings.

34

35   **Example 1:PKB influences GSK3 activity.**

36   *Fig 1:* a, L6 myotubes were incubated for 15 min with 2

1   mM 8-bromocyclic-AMP (8Br-cAMP) and then with 0.1 μM

2   insulin (5 min). Both GSK3 isoforms were co-

3   immunoprecipitated from the lysates and assayed before

4   (black bars) and after (white bars) reactivation with

5   PP2A (Cross et al., 1994). The results are presented

6   relative to the activity in unstimulated cells, which

7   was 0.08±0.006 U mg^{-1} (n=10).

8   b, c, The inhibition of GSK3 by insulin (0.1 μM) is

9   unaffected by rapamycin (0.1 μM) and PD 98059 (50 μM),

10   but prevented by LY 294002 (100 μM).

11

12   b, L6 myotubes were stimulated with insulin for the

13   times indicated with (filled triangle) or without

14   (filled circles) a 15 min preincubation with LY 294002,

15   and GSK3 measured as in a. The open circles show

16   experiments from insulin-stimulated cells where GSK3

17   was assayed after reactivation with PP2A (Cross et al.,

18   1994).

19

20   c, cells were incubated with rapamycin (triangles) or

21   rapamycin plus PD 98059 (circles) before stimulation

22   with insulin, and GSK3 activity measured as in a,

23   before (filled symbols) and after (open symbols)

24   pretreatment with PP2A.

25

26   d, e, L6 myotubes were incubated with 8Br-cAMP (15 min)

27   PD 98059 (60 min) or LY 294002 (15 min) and then with

28   insulin (5 min) as in a-c. Each enzyme was assayed

29   after immunoprecipitation from lysates, and the results

30   are presented relative to the activities obtained. In

31   the presence of insulin and absence of 8Br-cAMP, which

32   were 0.04±0.005 U mg^{-1} (p42 MAP kinase, n=6) and 0.071 ±

33   0.004 U mg^{-1} (MAPKAP Kinase^{-1}, n=6).

34

35   All the results (± s.e.m.) are for at least three

36   experiments.

1   Monolayers of L6 cells were cultured, stimulated and
2   lysed as described previously (Cross et al., 1994).
3   p42 MAP kinase, MAPKAP kinase 1 or (GSK3-α plus GSK3-β)
4   were then immunoprecipitated from the lysates and
5   assayed with specific protein or peptide substrates as
6   described previously (Cross et al., 1994). One unit of
7   protein kinase activity was that amount which catalysed
8   the phosphorylation of 1 nmol of substrate in 1 min.
9   Where indicated, GSK3 in immunoprecipitates was
10 reactivated with PP2A (Cross et al., 1994).
11
12
13 **Figure 2 Identification of PKB as the insulin-**
14 **stimulated, wortmannin-sensitive and PD**
15 **98059/rapamycin-insensitive Crosstide kinase in L6**
16 **myotubes.**
17 a. Cells were incubated with 50 μM PD 98059 (for 1
18 hour) and 0.1 μM rapamycin (10 min), then stimulated
19 with 0.1 μM insulin (5 min) and lysed (Cross et al.,
20 1994). The lysates (0.3 mg protein) were
21 chromatographed on Mono Q (5 x 0.16cm) and fractions
22 (0.05ml) were assayed for Crosstide kinase (filled
23 circles). In separate experiments insulin was omitted
24 (open circles) or wortmannin (0.1 μM) added 10 min
25 before the insulin (filled triangles). The broken line
26 shows the NaCl gradient. Similar results were obtained
27 in six experiments.
28
29 b. Pooled fractions (10 μl) 31-34 (lane 1), 35-38 (lane
30 2), 39-42 (lane 3), 43-45 (lane 4), 46-49 (lane 5) and
31 50-53 (lane 6) from a were electrophoresed on a 10%
32 SDS/polyacrylamide gel and immunoblotted with the C-
33 terminal anti-PKBα antibody. Marker proteins are
34 indicated. No immunoreactive species were present in
35 fractions 1-30 or 54-80.
36

1   *c.* L6 myotubes were stimulated with 0.1 μM insulin and

2   PKB immunoprecipitated from the lysates (50 μg protein)

3   essentially as described previously (Lazar et al.,

4   1995), using the anti-PH domain antibody and assayed

5   for Crosstide kinase (open circles). In control

6   experiments, myotubes were incubated with 0.1 μM

7   rapamycin plus 50 μM PD 98059 (open triangles) or 2 mM

8   8Br-cAMP (open squares), or 0.1 μM wortmannin (filled

9   circles) or 100 μM LY 294002 (filled triangles) before

10   stimulation with insulin.

11

12   *d.* As *c*, except that MAPKAP kinase-1 was

13   immunoprecipitated from the lysates and assayed with S6

14   peptide (filled circles). In control experiments,

15   cells were incubated with 0.1 μM rapamycin plus 50 μM

16   PD 98059 (filled triangles) or with 2 μM 8BR-cAMP (open

17   circles) before stimulation with insulin. In *c* and *d*,

18   the error bars denote triplicate determinations, and

19   similar results were obtained in three separate

20   experiments.

21

22   Mono Q chromatography was performed as described

23   (Burgering et al., 1995), except that the buffer also

24   contained 1 mM EGTA, 0.1 mM sodium orthovanadate and

25   0.5% (w/v) Triton X-100. Two PKBα antibodies were

26   raised in rabbits against the C-terminal peptide

27   FPQFSYSASSTA and bacterially expressed PH domain of

28   PKBα. The C-terminal antibody was affinity purified

29   (Jones et al., 1991). The activity of PKB towards

30   Crosstide is threefold higher than its activity towards

31   histone H2B and 11-fold higher than its activity

32   towards myelin basic protein, the substrates used

33   previously to assay PKB. Other experimental details

34   and units of protein kinase activity are given in

35   Fig 1.

36

1   **Figure 3 GSK3 is inactivated by PKB from insulin-**
2   **stimulated L6 myotubes.**
3   *a.* Cells were stimulated for 5 min with 0.1 μM insulin,
4   and PKB immunoprecipitated from 100 μg of cell lysate
5   and used to inactivate GSK3 isoforms essentially as
6   described previously (Sutherland et al., 1993 and
7   Sutherland et al., 1994). The black bars show GSK3
8   activity measured after incubation with MgATP and PKB
9   as a percentage of the activity obtained in control
10 incubations where PKB was omitted. In the absence of
11 PKB, GSK3 activity was stable throughout the
12 experiment. The white bars show the activity obtained
13 after reactivation of GSK3 with PP2A (Embi et al.,
14 1980). No inactivation of GSK3 occurred if insulin was
15 omitted, or if wortmannin (0.1 μM) was added 10 min
16 before the insulin, or if the anti-PKB antibody was
17 incubated with peptide immunogen (0.5 mM) before
18 immunoprecipitation. The results ($\pm$ s.e.m.) are for
19 three experiments (each carried out in triplicate).
20
21 *b.* Inactivation of GSK3-β by HA-PKBα. Complementary
22 DNA encoding HA-PKBα was transfected into COS-1 cells,
23 and after stimulation for 15 min with 0.1 mM sodium
24 pervanadate the tagged protein kinase was
25 immunoprecipitated from 0.3 mg of lysate and incubated
26 for 20 min with GSK3-β and MgATP. In control
27 experiments, pervanadate was omitted, or wildtype (WT)
28 PKBα replaced by vector (mock translation) or by a
29 kinase-inactive mutant of PKBα in which Lys 179 was
30 mutated to Ala (K179A). Similar results were obtained
31 in three separate experiments. The levels of WT and
32 K179A-PKBα in each immunoprecipitate were similar in
33 each transfection.
34
35 In *a* GSK3-α and GSK3-β were partially purified,
36 assayed, inactivated by PKB, and reactivated by PP2A

1   from rabbit skeletal muscle as described previously

2   (Sutherland et al., 1993 and Sutherland et al.,

3   1994).   There was no reactivation in control

4   experiments in which okadaic acid (2 μM) was added

5   before PP2A.

6

7   **Figure 4: Identification of the residues in GSK3**

8   **phosphorylated by PKB _in vitro_ and in response to**

9   **insulin in L6 myotubes.**

10   a. GSK3-β was maximally inactivated by incubation with

11   PKB and Mg-[γ-^{32}P]ATP and after SDS-PAGE, the ^{32}P-

12   labelled GSK3-β (M_r 47K) was digested with trypsin[11] and

13   chromatographed on a C_{18} column (Sutherland et al.,

14   1993).   Fractions (0.8 ml) were analysed for ^{32}P-

15   radioactivity (open circles), and the diagonal line

16   shows the acetonitrile gradient.

17

18   b. The major phosphopeptide from a (400 c.p.m.) was

19   subjected to solid-phase sequencing (Sutherland et al.,

20   1993), and ^{32}P-radioactivity released after each cycle

21   of Edman degradation is shown.

22

23   c. GSK3-α and GSK3-β were co-immunoprecipitated from

24   the lysates of ^{32}P-labelled cells, denatured in SDS,

25   subjected to SDS-PAGE, transferred to nitrocellulose

26   and autoradiographed (Saito et al., 1994).   Lanes 1-3,

27   GSK3 isoforms immunoprecipitated from unstimulated

28   cells; lanes 4-6, GSK3 isoforms immunoprecipitated from

29   insulin-stimulated cells. ↖

30

31   d. GSK3 isoforms from c. were digested with trypsin,

32   and the resulting phosphopeptides separated by

33   isoelectric focusing (Saito et al., 1994) and

34   identified by auto-radiography.   Lanes 1 and 4 show the

35   major phosphopeptide resulting from _in vitro_

36   phosphorylation of GSK3-β by PKB and MAPKAP kinase-1,

1 respectively; lanes 2 and 5, the phosphopeptides
2 obtained from GSK3-β and GSK3-α, immunoprecipitated
3 from unstimulated cells; lanes 3 and 6, the
4 phosphopeptides obtained from GSK3-β and GSK3-α
5 immunoprecipitated from cells stimulated for 5 min with
6 0.1 μM insulin; the arrow denotes the peptides whose
7 phosphorylation is increased by insulin. The pI values
8 of two markers, Patent Blue (2.4) and azurin (5.7) are
9 indicated.
10
11 In a. PKBα was immunoprecipitated with the C-terminal
12 antibody from the lysates (0.5 mg protein) of insulin-
13 stimulated L6 myotubes and used to phosphorylate GSK-
14 β[12]. In c. three 10-cm diameter dishes of L6 myotubes
15 were incubated for 4 hours in HEPES-buffered saline
16 (Cross et al., 1994) containing 50 μM PD 98059, 100 nM
17 rapamycin and 1.5 mCI ml^{-1} ^{32}P-orthophosphate, stimulated
18 for 5 min with insulin (0.1 μM) or buffer, and GSK3
19 isoforms co-immunoprecipitated from the lysates as in
20 Fig 1.
21
22 **Discussion.**
23 Inhibition of GSK3 induced by insulin in L6 myotubes
24 (Fig 1a-c) was unaffected by agents which prevented the
25 activation of MAPKAP kinase-1 (8-bromo-cyclic AMP, or
26 PD 98059 (Alessi et al., 1995), (Fig 1d,e) and/or p70^{S6k}
27 (rapamycin (Kuo et al., 1992)) (Cross et al., 1994),
28 suggesting that neither MAPKAP kinase-1 nor p70^{S6k} are
29 essential for this process. However, the
30 phosphorylation and inhibition of GSK3-β after phorbol
31 ester treatment (Stambolic et al., 1994) is enhanced by
32 coexpression with MAPKAP kinase 1 in HeLa S3 cells,
33 whereas in NIH 3T3 cells the EGF-induced inhibition of
34 GSK3-α and GSK3-β (Saito et al., 1994) is largely
35 suppressed by expression of a dominant-negative mutant
36 of MAP kinase kinase-1 (Elgar et al., 1995). MAPKAP

15

1   kinase-1 may therefore mediate the inhibition of GSK3
2   by agonists which are much more potent activators of
3   the classical MAP kinase pathway than is insulin.
4
5   To identify the insulin-stimulated protein kinase that
6   inhibits GSK3 in the presence of rapamycin and PD
7   98059, L6 myotubes were incubated with both compounds
8   and stimulated with insulin. The lysates were then
9   chromatographed on Mono Q and the fractions assayed
10   with "Crosstide" (GRPRTSSFAEG), a peptide corresponding
11   to the sequence in GSK3 surrounding the serine
12   (underlined) phosphorylated by MAPKAP kinase-1 and p70^{S6k}
13   (Ser 21 in GSK3-α (Sutherland et al., 1994) and Ser 9
14   in GSK3-β (Sutherland et al 1993)). Three peaks of
15   Crosstide kinase activity were detected, which were
16   absent if insulin stimulation was omitted or if the
17   cells were first preincubated with the PI 3-kinase
18   inhibitor wortmannin (Fig 2a). Wortmannin (Cross et
19   al., 1994 and Welsh et al 1994), and the structurally
20   unrelated PI 3-kinase inhibitor LY 294002 (ref 19);
21   (Fig 1b), both prevent the inhibition of GSK3 by
22   insulin.
23
24   The protein kinases PKB-α, PKB-β and PKBγ are Ser/Thr-
25   specific and cellular homologues of the viral oncogene
26   v-akt (Coffer et al., 1991, Jones et al 1991, Ahmed et
27   al 1995 and Cheng et al., 1992). These enzymes have
28   recently been shown to be activated in NIH 3T3, Rat-1
29   or Swiss 3T3 cells in response to growth factors or
30   insulin, activation being suppressed by blocking the
31   activation of PI 3-kinase in different ways (Franke et
32   al., 1995 and Burgering et al., 1995). All three peaks
33   of Crosstide kinase (Fig 2a), but no other fraction
34   from Mono Q, showed the characteristic multiple bands
35   of PKB (relative molecular mass, M$_r$ 58K, 59K or 60K)
36   that have been observed in other cells, when

1   immunoblotting was performed with an antibody raised

2   against the carboxyl-terminal peptide of PKB-α (Fig

3   2b).   The more slowly migrating forms represent more

4   highly phosphorylated protein, and are converted to the

5   fastest migrating species by treatment with

6   phosphatases.   Phosphatase treatment also results in

7   the inactivation of PKB (Burgering et al., 1995) and

8   the complete loss of Crosstide kinase activity (data

9   not shown).   Of the Crosstide kinase activity in peaks

10   2 and 3 from Mono Q, 70-80% was immunoprecipitated by a

11   separate antibody raised against the amino-terminal

12   pleckstrin homology (PH) domain of PKB-α.   The C-

13   terminal antibody also immunoprecipitated PKB activity

14   specifically from peaks 2 and 3, but was less effective

15   than the anti-PH-domain antibody.   Peak-1 was hardly

16   immunoprecipitated by either antibody and may represent

17   PKB-β.   An immunoprecipitating anti-MAPKAP kinase-1

18   antibody (Cross et al., 1994) failed to deplete any of

19   the Crosstide kinase activity associated with peaks 1,

20   2 or 3.

21

22   Insulin stimulation of L6 myotubes increased PKB

23   activity by more than tenfold (Fig 2c), and activation

24   was blocked by wortmannin or LY 294002, but was

25   essentially unaffected by 8-bromo-cyclic AMP or

26   rapamycin plus PD 98059 (Fig 2c).   The half-time ($t_{0.5}$)

27   or activation of PKB (1 min) was slightly faster than

28   that for inhibition of GSK3 (2 min) (Cross et al.,

29   1994).   In contrast, the activation of MAPKAP kinase-1

30   (Fig 2d) and $p70^{S6k}$ (not shown) was slower ($t_{0.5} > 5$ min).

31   Activation of MAPKAP kinase-1 was prevented by 8-bromo-

32   cyclic AMP or PD 98059 (Fig 2d), and activation of $p70^{S6k}$

33   by rapamycin (Cross et al., 1994).   Akt/RAC

34   phosphorylated the Ser in the Crosstide equivalent to

35   Ser 21 in GSK3-α and Ser 9 in GSK3-β (data not shown).

36

1   PKB from insulin-stimulated L6 myotubes (but not from
2   unstimulated or wortmannin-treated cells) inactivated
3   GSK3-α and GSK3-β *in vitro*, and inhibition was reversed
4   by the Ser/Thr-specific protein phosphatase PP2A (Embi
5   et al., 1980) (Fig 3a). To further establish that
6   inactivation was catalysed by PKB, and not by a co-
7   immunoprecipitating protein kinase, haemagglutonin-
8   tagged PKB-α (HA-PKB) was transfected into COS-1 cells
9   and activated by stimulation with pervanadate, which is
10 the strongest inducer of PKB activation in this system.
11 The HA-PKB inactivated GSK3-β, but not if treatment
12 with pervanadate was omitted or if wild-type HA-PKB was
13 replaced with a "kinase inactive" mutant (Fig 3b).
14
15 The inactivation of GSK3-β by PKB *in vitro* was
16 accompanied by the phosphorylation of one major tryptic
17 peptide (Fig 4a) which coeluted during C_{18}
18 chromatography (Sutherland et al., 1993) and
19 isoelectric focusing with that obtained after
20 phosphorylation by MAPKAP kinase-1 (Fig 4d).
21 Stimulation of L6 myotubes with insulin (in the
22 presence of rapamycin and PD 98059) increased the ^{32}P-
23 labelling of GSK3-α and GSK3-β by 60-100% (Fig 4c) and
24 increased the ^{32}P-labelling of the same tryptic peptides
25 labelled *in vitro* (Fig 4d). Sequence analyses
26 established that the third residue of these,
27 corresponding to Ser 9 (GSK3-β) or Ser 21 (GSK3-α), was
28 the site of phosphorylation in each phosphopeptide,
29 both *in vitro* (Fig 4b) and *in vivo* (not shown). The
30 ^{32}P-labelling of other (more acidic) tryptic
31 phosphopeptides was not increased by insulin (Fig 4d).
32 These peptides have been noted previously in GSK3 from
33 A431 cells and shown to contain phosphoserine and
34 phosphotyrosine (Saito et al., 1994).
35
36 PKC-δ, ε and ζ are reported to be activated by

1   mitogens, and PKC-ζ activity is stimulated *in vitro* by
2   several inositol phospholipids, including PI(3,4,5)P_3
3   the product of the PI 3-kinase reaction (Andjelkovic et
4   al., 1995). However, purified PKC-ε (Palmer et al.,
5   1995), PKC-δ and PKC-ζ (data not shown) all failed to
6   inhibit GSK3-α or GSK3-β *in vitro*. Moreover, although
7   PKC-α, β1 and γ inhibit GSK3-β *in vitro* (Palmer et al.,
8   1995), GSK3-α is unaffected, while their downregulation
9   in L6 myotubes by prolonged incubation with phorbol
10   esters abolishes the activation of MAPKAP kinase-1 in
11   response to subsequent challenge with phorbol esters,
12   but has no effect on the inhibition of GSK3 by insulin
13   (not shown).
14
15   Taken together, our results identify GSK3 as a
16   substrate for PKB. The stimulation of glycogen
17   synthesis by insulin in skeletal muscle involves the
18   dephosphorylation of Ser residues in glycogen synthase
19   that are phosphorylated by GSK3 *in vitro* (Parker et
20   al., 1983). Hence the 40-50% inhibition of GSK3 by
21   insulin, coupled with a similar activation of the
22   relevant glycogen synthase phosphatase (Goode et al.,
23   1992), can account for the stimulation of glycogen
24   synthase by insulin in skeletal muscle (Parker et al.,
25   1983) or L6 myotubes (Goode et al., 1992). The
26   activation of glycogen synthase and the resulting
27   stimulation of glycogen synthesis by insulin in L6
28   myotubes is blocked by wortmannin, but not by PD 98059
29   (Dent et al., 1990), just like the activation of
30   Akt/RAC and inhibition of GSK3. However, GSK3 is
31   unlikely to be the only substrate of PKB *in vivo*, and
32   identifying other physiologically relevant substrates
33   will be important because PKBβ is amplified and over-
34   expressed in many ovarian neoplasms (Cheng et al.,
35   1992).
36

1   **Example 2: Activation of PKB by insulin in L6 myotubes**

2   **is accompanied by phosphorylation of residues Thr-308**

3   **and Ser-473.** Insulin induces the activation and

4   phosphorylation of PKBα in L6 myotubes. Three 10 cm

5   dishes of L6 myotubes were ^{32}P-labelled and treated for

6   10 min with or without 100 nM wortmannin and then for 5

7   min with or without 100 nM insulin. PKBα was

8   immunoprecipitated from the lysates and an aliquot

9   (15%) assayed for PKBα activity (Fig 5A). The

10   activities are plotted ± SEM for 3 experiments relative

11   to PKBα derived from unstimulated cells which was 10

12   mU/mg. The remaining 85% of the immunoprecipitated PKBα

13   was alkylated with 4-vinylpyridine, electrophoresed on

14   a 10% polyacrylamide gel (prepared without SDS to

15   enhance the phosphorylation-induced decrease in

16   mobility) and autoradiographed. The positions of the

17   molecular mass markers glycogen phosphorylase (97 kDa),

18   bovine serum albumin (66 kDa) and ovalbumin (43 kDa)

19   are marked.

20

21   Under these conditions, insulin stimulation resulted in

22   a 12-fold activation of PKBα (Fig 5A) and was

23   accompanied by a 1.9 ± 0.3-fold increase in

24   ^{32}P-labelling (4 experiments) and retardation of its

25   mobility on SDS-polyacrylamide gels (Fig 5B). The

26   activation of PKBα, the increase in its ^{32}P-labelling

27   and reduction in electrophoretic migration were all

28   abolished by prior incubation of the cells with 100 nM

29   wortmannin. Phosphoamino acid analysis of the whole

30   protein revealed that ^{32}P-labelled PKBα was

31   phosphorylated at both serine and threonine residues

32   and that stimulation with insulin increased both the

33   ^{32}P-labelling of both phosphoamino acids (data not

34   shown).

35

36   **Fig. 6. Insulin stimulation of L6 myotubes induces the**

1   **phosphorylation of two peptides in PKBα.**   Bands

2   corresponding to ^{32}P-labelled PKBα from Fig 5B were

3   excised from the gel, treated with 4-vinylpyridine to

4   alkylate cysteine residues, digested with trypsin and

5   chromatographed on a Vydac 218TP54 C18 column

6   (Separations Group, Hesperia, CA) equilibrated with

7   0.1% (by vol) trifluoroacetic acid (TFA), and the

8   columns developed with a linear acetonitrile gradient

9   (diagonal line). The flow rate was 0.8 ml / min and

10   fractions of 0.4 ml were collected (A) tryptic peptide

11   map of ^{32}P-labelled PKBα from unstimulated L6 myotubes;

12   (B) tryptic peptide map of ^{32}P-labelled PKBα from

13   insulin-stimulated L6 myotubes; (C) tryptic peptide map

14   of ^{32}P-labelled PKBα from L6 myotubes treated with

15   wortmannin prior to insulin. The two major ^{32}P-labelled

16   peptides eluting at 23.7% and 28% acetonitrile are

17   named Peptide A and Peptide B, respectively. Similar

18   results were obtained in four (A and B) and two (C)

19   experiments.

20

21   No major ^{32}P-labelled peptides were recovered from

22   ^{32}P-labelled PKBα derived from unstimulated L6 myotubes

23   (Fig 6A) indicating that, in the absence of insulin,

24   there was a low level phosphorylation at a number of

25   sites. However, following stimulation with insulin, two

26   major ^{32}P-labelled peptides were observed, termed A and

27   B (Fig 6B), whose ^{32}P-labelling was prevented if the

28   myotubes were first preincubated with wortmannin (Fig

29   6C).

30

31   **Fig 7. Identification of the phosphorylation sites in**

32   **peptides A and B.** (A) Peptides A and B from Fig5B

33   (1000cpm) were incubated for 90min at 110°C in 6M HCl,

34   electrophoresed on thin layer cellulose at pH 3.5 to

35   resolve orthophosphate (Pi), phosphoserine (pS),

36   phosphthreonine (pT) and phosphotyrosine (pY) and

1   autoradiographed. (B) Peptide A (Fig 5B) obtained from

2   50 10 cm dishes of ^{32}P-labelled L6 myotubes was further

3   purified by chromatography on a microbore C18 column

4   equilibrated in 10 mM ammonium acetate pH 6.5 instead

5   of 0.1% TFA. A single peak of ^{32}P-radioactivity was

6   observed at 21% acetonitrile which coincided with a

7   peak of 214 nm absorbance. 80% of the sample (1 pmol)

8   was analysed on an Applied Biosystems 476A sequencer to

9   determine the amino acid sequence, and the

10   phenylthiohydantoin (Pth) amino acids identified after

11   each cycle of Edman degradation are shown using the

12   single letter code for amino acids. The residues in

13   parentheses were not present in sufficient amounts to

14   be identified unambiguously. To identify the site(s)

15   of phosphorylation, the remaining 20% of the sample

16   (600 cpm) was then coupled covalently to a Sequelon

17   arylamine membrane and analysed on an Applied

18   Biosystems 470A sequencer using the modified programme

19   described by Stokoe et al. (1992). ^{32}P radioactivity was

20   measured after each cycle of Edman degradation. (C)

21   Peptide B from Fig 2B (800 cpm) was subjected to solid

22   phase sequencing as in (B).

23

24   Peptide A was phosphorylated predominantly on serine

25   while peptide B was labelled on threonine (Fig 7A).

26   Amino acid sequencing established that peptide A

27   commenced at residue 465. Only a single burst of

28   ^{32}P-radioactivity was observed after the eighth cycle of

29   Edman degradation (Fig 7B), demonstrating that insulin

30   stimulation of L6 myotubes had triggered the

31   phosphorylation of PKBα at Ser-473, which is located 9

32   residues from the C-terminus of the protein.

33   Phosphopeptide B was only recovered in significant

34   amounts if ^{32}P-labelled PKBα was treated with

35   4-vinylpyridine prior to digestion with trypsin,

36   indicating that this peptide contained a cysteine

1   residue(s), and a single burst of 32p radioactivity was
2   observed after the first cycle of Edman degradation
3   (Fig 7C). This suggested that the site of
4   phosphorylation was residue 308, since it is the only
5   threonine in PKBa that follows a lysine or arginine
6   residue and which is located in a tryptic peptide
7   containing a cysteine residue (at position 310). The
8.   acetonitrile concentration at which phosphopeptide B is
9   eluted from the C18 column (28%) and its isoelectric
10   point (4.0) are also consistent with its assignment as
11   the peptide comprising residues 308-325 of PKBα. The
12   poor recoveries of Peptide B during further
13   purification at pH 6.5 prevented the determination of
14   its amino acid sequence, but further experiments
15   described below using transiently transfected 293 cells
16   established that this peptide does correspond to
17   residues 308-325 of PKBα.

18

19   **Fig 8: Mapping the phosphorylation sites of PKBα in**
20   **transiently transfected 293 cells.** 293 cells were
21   transiently transfected with DNA constructs expressing
22   wild type PKBα, or a haemagglutonin epitope-tagged PKBα
23   encoding the human protein, such as HA-KD PKBα, HA-473A
24   PKBα or HA-308A PKBα. After treatment for 10 min with
25   or without 100 nM wortmannin, the cells were stimulated
26   for 10 min with or without 100 nM insulin or 50 ng/ml
27   IGF- 1 in the continued presence of wortmannin. PKBα
28   was immunoprecipitated from the lysates and assayed,
29   and activities corrected for the relative levels of
30   expression of each HA-PKBα. The results are expressed
31   relative to the specific activity of wild type HA-PKBα
32   from unstimulated 293 cells (2.5 ± 0.5 U/mg). (B) 20 μg
33   of protein from each lysate was electrophoresed on a 10
34   % SDS/polyacrylamide gel and immunoblotted using
35   monoclonal HA-antibody. The molecular markers are those
36   used in Fig 5B.

1   **Fig 9: IGF-1 stimulation of 293 cells induces the**
2   **phosphorylation of two peptides in transfected HA-PKBα.**
3   293 cells transiently transfected with wild type HAPKBα
4   DNA constructs were ^{32}P-labelled, treated for 10 min
5   without (A,B) or with (C) 100 nM wortmannin and then
6   for 10 min without (A) or with (B, C) 50 ng/ml IGF-1.
7   The 32p labelled HA-PKBα was immunoprecipitated from
8   the lysates, treated with 4-vinylpyridine,
9   electrophoresed on a 10% polyacrylamide gel, excised
10   from the gel and digested with trypsin. Subsequent
11   chromatography on a C_{18} column resolved four major
12   phosphopeptides termed C, D, E and F. Similar results
13   were obtained in 6 separate experiments for (A) and
14   (B), and in two experiments for (C).
15
16   Stimulation with insulin and IGF-1 resulted in 20-fold
17   and 46-fold activation of transfected PKBα,
18   respectively (Fig 8A), the half time for activation
19   being 1 min, as found with other cells. Activation of
20   PKBα by insulin or IGF-1 was prevented by prior
21   incubation with wortmannin (Fig 8A) and no activation
22   occurred if 293 cells were transfected with vector
23   alone and then stimulated with insulin or 1GF-1 (data
24   not shown).
25
26   Two prominent ^{32}P-labelled peptides were present in
27   unstimulated 293 cells (Fig 9A). One, termed Peptide C,
28   usually eluted as a doublet at 20-21% acetonitrile
29   while the other, termed Peptide F, eluted at 29.7%
30   acetonitrile. Stimulation with insulin or IGF-1 did
31   not affect the ^{32}P-labelling of Peptides C and F (Figs
32   9A & B), but induced the ^{32}P-labelling of two new
33   peptides, termed D (23.4% acetonitrile) and E
34   (28% acetonitrile), which eluted at the same
35   acetonitrile concentrations as peptides A and B from L6
36   myotubes (Fig 6B) and had the same isoelectric points

24

1   (7.2 and 4.0, respectively). Treatment of 293 cells

2   expressing HA-PKBα with 100 nM wortmannin, prior to

3   stimulation with IGF-1, prevented the phosphorylation

4   of Peptides D and E, but had no effect on the 32p

5   labelling of Peptides C and F (Fig 9C).

6

7   Peptides C, D, E and F were further purified by re-

8   chromatography on the C18 column at pH 6.5 and

9   sequenced. Peptides C gave rise to three separate (but

10   closely eluting) ^{32}P-labelled peptides (data not shown).

11   Amino acid sequencing revealed that all three commenced

12   at residue 122 of PKBα and that Ser-124 was the site of

13   phosphorylation (Fig 10A). Peptide D only contained

14   phosphoserine and, as expected, corresponded to the

15   PKBα tryptic peptide commencing at residue 465 that was

16   phosphorylated at Ser-473 (Fig 10B). Peptide E, only

17   contained phosphothreonine and amino acid sequencing

18   demonstrated that it corresponded to residues 308-325,

19   the phosphorylation site being Thr-308 (Fig 10C).

20   Peptide F only contained phosphothreonine and

21   corresponded to the peptide commencing at residue 437

22   of PKBα phosphorylated at Thr-450 (Fig 10D).

23

24   In the presence of phosphatidylserine, PKBα binds to

25   PIP3 with submicromolar affinity (James et al., 1996,

26   Frech et al.,1996). Phosphatidyl 4,5-bisphosphate and

27   phosphatidyl 3,4 bisphosphate bind to PKBα with lower

28   affinities and PI 3,5 bisphosphate and PI 3 phosphate

29   did not bind at all under these conditions (James et

30   al., 1996). The region of PKBα that interacts with PIP3

31   is almost certainly the PH domain, because the isolated

32   PH domain binds PIP3 with similar affinity to PKBα

33   itself (Frech et al., 1996) and because the PH domain

34   of several other proteins, such as the PH-domains of,

35   β-spectrin and phospholipase C1, are known to interact

36   specifically with other phosphoinositides (Hyvonen et

1   al., 1995 and Lemmon et al., 1995).

2

3   The experiments described above were repeated using

4   insulin instead of IGF-1. The results were identical,

5   except that the ^{32}P-labelling of Peptides D and E was

6   about 50% of the levels observed with IGF-1 (data not

7   shown). This is consistent with the two-fold lower

8   level of activation of PKBα by insulin compared with

9   IGF-1 (Fig 7A).

10

11   **Example 3: MAPKAP kinase-2 phosphorylates Ser-473 of**

12   **PKBα causing partial activation.** Ser-473 of PKBa lies

13   in a consensus sequence Phe-x-x-Phe/Tyr-Ser/Thr-Phe/Tyr

14   found to be conserved in a number of protein kinases

15   that participate in signal transduction pathways

16   (Pearson et al. 1995). In order to identify the Ser-473

17   kinase(s) we therefore chromatographed rabbit skeletal

18   muscle extracts on CM-Sephadex, and assayed the

19   fractions for protein kinases capable of

20   phosphorylating a synthetic peptide corresponding to

21   residues 465 to 478 of PKBα. These studies identified

22   an enzyme eluting at 0.3 M NaCl which phosphorylated

23   the peptide 465-478 at the residue equivalent to

24   Ser-473 of PKBα. The Ser473 kinase co-eluted from

25   CM-Sephadex with MAP kinase-activated protein (MAPKAP)

26   kinase-2, (Stokoe et al, 1992) which is a component of

27   a stress and cytokine-activated MAP kinase cascade

28   (Rouse et al, 1994; Cuenda et al, 1995). The Ser-473

29   kinase continued to cofractionate with MAPKAPkinase-2

30   through phenyl-Sepharose, heparin-Sepharose, Mono S and

31   Mono Q and was immunoprecipitated quantitatively by an

32   anti-MAPKAP kinase-2 antibody (Gould et al, 1995)

33   demonstrating that MAPKAP kinase-2 was indeed the

34   Ser-473 kinase we had purified.

35

36   **Figure 11.** HA-PKBα was immunoprecipitated from the

26

1   lysates of unstimulated COS-1 cells expressing these
2   constructs. (A) 0.5 μg of immunoprecipitated HA-PKBα
3   was incubated with MAPKAP kinase-2 (50 U/ml), 10 mM
4   magnesium acetate and 100 mM [γ^{32}P]ATP in a total of 40
5   μl of Buffer B. At various times, aliquots were removed
6   and either assayed for PKBα activity (open circles) or
7   for incorporation of phosphate into PKBα (closed
8   circles). Before measuring PKBa activity, EDTA was
9   added to a final concentration of 20 mM to stop the
10   reaction, and the immunoprecipitates washed twice with
11   1.0 ml of buffer B containing 0.5 M NaCl, then twice
12   with 1.0 ml of Buffer B to remove MAPKAP kinase-2. The
13   results are presented as $\pm$ SEM for six determinations
14   (two separate experiments) and PKBα activities are
15   presented relative to control experiments in which
16   HA-PKBα was incubated with MgATP in the absence of
17   MAPKAP kinase-2 (which caused no activation).
18   Phosphorylation was assessed by counting the
19   ^{32}P-radioactivity associated with the band of PKBα after
20   SDS/polyacrylamide gel electrophoresis. The open
21   triangles show the activity of immunoprecipitated HA-KD
22   PKBα phosphorylated by MAPKAP kinase-2. (B) HA-PKBα
23   phosphorylated for 1 h with MAPKAP kinase-2 and ^{32}P-
24   γ-ATP as in (A) was digested with trypsin and
25   chromatographed on a C18 column as described in the
26   legend for Fig 2. (C) The major ^{32}P-labelled peptide
27   from (B) was analysed on the 470A sequencer as in Fig 3
28   to identify the site of phosphorylation.
29
30   Bacterially expressed MAPKAP kinase-2 phosphorylated
31   wild type HA-PKBα or the catalytically inactive mutant
32   HA-PKBα in which Lys- 179 had been mutated to Ala (data
33   not shown) to a level approaching 1 mol per mole
34   protein (Fig 11A). Phosphorylation of wild-type PKBα
35   was paralleled by a seven-fold increase in activity,
36   whereas phosphorylation of the catalytically inactive

mutant did not cause any activation (Fig 11A). No
phosphorylation or activation of wild type HA-PKBα
occurred if MAPKAP kinase-2 or MgATP was omitted from
the reaction (data not shown). Wild type HA-PKBα that
had been maximally activated with MAPKAP kinase-2, was
completely dephosphorylated and inactivated by
treatment with protein phosphatase 2A (data not shown).

HA-PKBα that had been maximally phosphorylated with
MAPKAP kinase-2 was digested with trypsin and C18
chromatography revealed a single major ^{32}P-labelled
phosphoserine-containing peptide (Fig 11B). This
peptide eluted at the same acetonitrile concentration
(Fig 11B) and had the same isoelectric point of 7.2
(data not shown) as the 32p labelled tryptic peptide
containing Ser-473 (compare Figs 11B and 6B). Solid
phase sequencing gave a burst of ^{32}P-radioactivity after
the eighth cycle of Edman degradation (Fig 11C),
establishing that Ser-473 was the site of
phosphorylation. The same ^{32}P-peptide was obtained
following tryptic digestion of catalytically inactive
HA-KD PKBα that had been phosphorylated with MAPKAP
kinase-2 (data not shown).

**Example 4: Phosphorylation of Thr-308 and Ser-473
causes synergistic activation of PKBα.** The experiments
described above demonstrated that phosphorylation of
Ser-473 activates PKBα in vitro but did not address the
role of phosphorylation of Thr-308, or how
phosphorylation of Thr-308 might influence the effect
of Ser-473 phosphorylation on activity, or vice versa.
We therefore prepared haemagglutonin (HA)-tagged PKBα
DNA constructs in which either Ser-473 or Thr-308 would
be changed either to Ala (to block the effect of
phosphorylation) or to Asp (to try and mimic the effect
of phosphorylation).

1   **Fig 12. Activation of HA-PKBα mutants in vitro by**
2   **MAPKAP kinase-2.** (A) Wild type and mutant HA-PKBα
3   proteins were immunoprecipitated from the lysates of
4   unstimulated COS-1 cells expressing these constructs
5   and incubated for 60 min with MgATP in the absence (-,
6   filled bars) or presence (+, hatched bars) of MAPKAP
7   kinase-2 and MgATP (50 U/ml). The PKBα protein was
8   expressed as similar levels in each construct and
9   specific activities are presented relative to wild type
10   HA-PKBα incubated in the absence of MAPKAP kinase-2
11   (0.03 U/mg). The results are shown as the average $\pm$ SEM
12   for 3 experiments. (B) 20 μg of protein from each
13   lysate was electrophoresed on a 10 % SDS/polyacrylamide
14   gel and immunoblotted using monoclonal HA-antibody.
15
16   All the mutants were expressed at a similar level in
17   serum-starved COS-1 cells (data not shown) and the
18   effects of maximally phosphorylating each of them at
19   Ser-473 is shown in Fig 12A. Before phosphorylation
20   with MAPKAP kinase-2 the activity of HA-473A PKBα was
21   similar to that of unstimulated wild type HA-PKBα and,
22   as expected, incubation with MAPKAP kinase-2 and MgATP
23   did not result in any further activation of HA-473A
24   PKBα. In contrast, the activity of HA-473D PKBα was
25   five-fold to six-fold higher than that of unstimulated
26   wild type HAPKBα protein, and similar to that of
27   wild-type HA-PKBα phosphorylated at Ser-473. As
28   expected, HA-473D PKBα was also not activated further
29   by incubation with MAPKAP kinase-2 and MgATP. The
30   activity of HA-308A PKBα was about 40% that of the
31   unstimulated wild type enzyme, and after
32   phosphorylation with MAPKAP kinase-2 is activity
33   increased to a level similar to that of wild type
34   HA-PKBα phosphorylated at Ser-473. Interestingly,
35   HA-308D PKBα which (like HA-473D PK) was five-fold more
36   active than dephosphorylated wild type HA-PKBα, was

1   activated dramatically by phosphorylation of Ser-473.
2   After incubation with MAPKAP kinase-2 and MgATP, the
3   activity of HA-308D PKBα was nearly five-fold higher
4   than that of wild type HA-PKBα phosphorylated at
5   Ser-473 (Fig 12B). These results suggested that the
6   phosphorylation of either Thr-308 or Ser-473 leads to
7   partial activation of PKBα in vitro, and that
8   phosphorylation of both residues results in a
9   synergistic activation of the enzyme. This idea was
10 supported by further experiments in which both Thr-308
11 and Ser-473 were changed to Asp. When this double
12 mutant was expressed in COS-1 cells it was found to
13 possess an 18-fold higher specific activity than the
14 dephosphorylated wild type protein. As expected, the
15 activity of this mutant was not increased further by
16 incubation with MAPKAP kinase-2 and MgATP (Fig 12B).
17
18 **Example 5:   Phosphorylation of both Thr-308 and Ser-473**
19 **is required for a high level of activation of PKBα in**
20 **vivo.**
21
22 **Fig 9. Effect of mutation of PKBα on its activation by**
23 **insulin in 293 cells.** (A) 293 cells were transiently
24 transfected with DNA constructs expressing wild type
25 PKBa, HA-D473- PKBα, and HA-308D/473D-PKBα. After
26 treatment for 10 min with or without 100 nM wortmannin,
27 cells were stimulated for 10 min with or without 100 nM
28 insulin in the continued presence of wortmannin. PKBα
29 was immunoprecipitated from the lysates and assayed,
30 and activities corrected for the relative levels of
31 HA-PKBα expression as described in the methods. The
32 results are expressed relative to the specific activity
33 of wild type HA-PKBα obtained from unstimulated 293
34 cells. (B) 20 μg of protein from each lysate was
35 electrophoresed on a 10 % SDS/polyacrylamide gel and
36 immunoblotted using monoclonal HA-antibody.

1   The basal level of activity of HA-473A PKBα derived

2   from unstimulated cells was similar to that of wild

3   type PKBα (Fig 8A). Stimulation of 293 cells expressing

4   HA-473A PKBα with insulin or IGF-1 increased the

5   activity of this mutant three-fold and five-fold

6   respectively; i.e. to 15% of the activity of wild type

7   HA-PKBα which had been transiently expressed and

8   stimulated under identical conditions. The basal

9   activity of HA-308A PKBα in unstimulated cells was also

10   similar to that of wild type HA-PKBα derived from

11   unstimulated cells, but virtually no activation of this

12   mutant occurred following stimulation of the cells with

13   insulin or IGF-1. These data are consistent with in

14   vitro experiments and indicate that maximal activation

15   of PKBα requires phosphorylation of both Ser-473 and

16   Thr-308 and that phosphorylation of both residues

17   results in a synergistic activation of the enzyme.

18   Consistent with these results, HA-473D PKBα displayed

19   five-fold higher activity and the HA-308D/HA473D double

20   mutant 40-fold higher activity than wild type HA-PKBα

21   when expressed in unstimulated cells. Following

22   stimulation with insulin, HA-473D PKBα was activated to

23   a level similar to that observed with the wildtype

24   enzyme, while the HA-308D/HA-473D double mutant could

25   not be activated further (Fig 13). As expected,

26   activation of HA-473D PKBα by insulin was prevented by

27   wortmannin, and the activity of the HA-308D/ HA-473D

28   double mutant was resistant to wortmannin (Fig 13).

29

30   **Example 6: Phosphorylation of Thr-308 is not dependent**

31   **on phosphorylation of Ser-473 or vice versa (in 293**

32   **cells).** (Fig 10) A 10 cm dish of 293 cells were

33   transfected with either HA-308A PKBα or HA-473A PKBα,

34   ^{32}P-labelled, then stimulated for 10 min with either

35   IGF-1 (50 ng/ml) or buffer. The ^{32}P-labelled PKBα

36   mutants were immunoprecipitated from the lysates,

1   treated with 4-vinylpyridine, electrophoresed on a 10%
2   polyacrylamide gel, excised from the gel and digested
3   with trypsin, then chromatographed on a C18 column.
4   The tryptic peptides containing the phosphorylated
5   residues Ser-124, Thr-308, Thr-450, Ser-473 are marked
6   and their assignments were confirmed by phosphoamino
7   acid analysis and sequencing to identify the sites of
8   phosphorylation (data not shown). The phosphopeptides
9   containing Thr-308 and Ser-473 were absent if
10 stimulation with IGF-1 was omitted, while the
11 phosphopeptides containing Ser-124 and Thr-450 were
12 present at similar levels as observed with wild-type
13 PKBα (see Fig 9A). Similar results were obtained in 3
14 separate experiments.
15
16 These experiments demonstrated that IGF-1 stimulation
17 induced the phosphorylation of HA-473A PKBα at Thr-308,
18 and the phosphorylation of HA-308A PKBα at Ser-473.
19 Similar results were obtained after stimulation with
20 insulin rather than IGF-I.
21
22 **Example 7:   IGF-1 or insulin induces phosphorylation of**
23 **Thr-308 and Ser-473 in a catalytically inactive mutant**
24 **of PKBα.**
25
26 **Fig 15. The catalytically inactive PKBα mutant**
27 **(HA-KD-PKBα) expressed in 293 cells is phosphorylated**
28 **at Thr-308 and Ser-473 after stimulation with IGF-1.**
29 Each 10 cm dish of 293 cells transiently transfected
30 with HA-KD-PKBα DNA constructs was ^{32}P-labelled and
31 incubated for 10 min with buffer (A), 50 ng/ml IGF-1
32 (B) or 100 nM insulin (C). The ^{32}P-labelled HA-KD-PKBα
33 was immunoprecipitated from the lysates, treated with 4
34 vinylpyridine, electrophoresed on a 10% polyacrylamide
35 gel, excised from the gel and digested with trypsin,
36 then chromatographed on a C18 column. The tryptic

1   peptides containing the phosphorylated residues
2   Ser-124, Thr-308, Thr-450 and Ser-473 are marked.
3   Similar results were obtained in 3 separate experiments
4   for (A) and (B), and in two experiments for (C).
5
6   This kinase dead" mutant of PKBα, termed HA-KD-PKBα, in
7   which Lys-179 was changed to Ala (see above) was
8   transiently expressed in 293 cells and its level of
9   expression found to be several-fold lower than that of
10   wild type HA-PKBα expressed under identical conditions
11   (Fig 8B). As expected, no PKBα activity was detected
12   when 293 cells expressing HA-KD-PKBα, were stimulated
13   with insulin or IGF-1 (Fig 7A).
14
15   293 cells that had been transiently transfected with
16   HA-KD-PKBα were ^{32}P-labelled, then stimulated with
17   buffer, insulin or IGF-1. and sites on PKBα
18   phosphorylated under these conditions were mapped. In
19   contrast to wild type HA-PKBα from unstimulated 293
20   cells (Fig 9), HA-KD PKBα was phosphorylated to a much
21   lower level at Ser-124, but phosphorylated similarly at
22   Thr-450 (Fig 15A). Following stimulation with IGF-1
23   (Fig 15B) or insulin (Fig 14C) HA-KD-PKBα became
24   phosphorylated at the peptides containing Thr-308 and
25   Ser-473, the extent of phosphorylation of these sites
26   being at least as high as wild type PKBα. Amino acid
27   sequencing of these peptides established that they were
28   phosphorylated at Thr-308 and Ser-473, respectively.
29
30   The above examples establish that PKB influences GSK3
31   activity; that Thr-308 and Ser-473 are the major
32   residues in PKBα that become phosphorylated in response
33   to insulin or IGF-1 (Figs 2 and 5) and that
34   phosphorylation of both residues is required to
35   generate a high level of PKBα activity. Thus mutation
36   of either Thr-308 or Ser-473 to Ala greatly decreased

1 the activation of transfected PKBα by insulin or IGF-1

2 in 293 cells (Fig 8). Moreover, PKBα became partially

3 active in vitro when either Thr-308 or Ser-473 were

4 changed to Asp or when Ser-473 was phosphorylated by

5 MAPKAP kinase-2 in vitro, and far more active when the

6 D308 mutant of PKBα was phosphorylated by MAPKAP

7 kinase-2 or when Thr-308 and Ser-473 were both mutated

8 to Asp (Fig 12). Moreover, the D308/D473 double mutant

9 could not be activated further by stimulating cells

10 with insulin (Fig 13). These observations demonstrate

11 that the phosphorylation of Thr-308 and Ser-473 act

12 synergistically to generate a high level of PKBα

13 activity.

14

15 Thr-308, and the amino acid sequence surrounding it, is

16 conserved in rat PKBβ and PKBγ but, interestingly,

17 Ser-473 (and the sequence surrounding it) is only

18 conserved in PKBβ. In rat PKBγ, Ser-473 is missing

19 because the C-terminal 23 residues are deleted. This

20 suggests that the regulation of PKBγ may differ

21 significantly from that of PKBα and PKBβ in the rat.

22

23 Thr-308 is located in subdomain VIII of the kinase

24 catalytic domain, nine residues upstream of the

25 conserved Ala-Pro-Glu motif, the same position as

26 activating phosphorylation sites found in many other

27 protein kinases. However, Ser-473 is located C-terminal

28 to the catalytic domain in the consensus sequence

29 Phe-Xaa-Xaa-Phe/Tyr-Ser/Thr-Phe/Tyr which is present in

30 several protein kinases that participate in growth

31 factor-stimulated kinase cascades, such as p70 S6

32 kinase, PKC and p90rsk (Pearson et al, 1995). However,

33 it is unlikely that a common protein kinase

34 phosphorylates this motif in every enzyme for the

35 following reasons. Firstly, phosphorylation of the

36 equivalent site in p70 S6 kinase is prevented by the

1   immunosuppressant drug rapamycin (Pearson et al, 1995)
2   which does not prevent the activation of PKBα by
3   insulin (Cross et al, 1995) or is phosphorylation at
4   Ser-473 (D. Alessi, unpublished work). Secondly, the
5   equivalent residue in protein kinase C is
6   phosphorylated constitutively and not triggered by
7   stimulation with growth factors (Tsutakawa et al.,
8   1995).
9
10   MAPKAP kinase-2 is a component of a protein kinase
11   cascade which becomes activated when cells are
12   stimulated with interleukin-1 or tumour necrosis factor
13   or exposed cellular stresses (Rouse et al, 1994; Cuenda
14   et al, 1995). MAPKAP kinase-2 phosphorylates PKBα
15   stoichiometrically at Ser-473 (Fig 11) and this finding
16   was useful in establishing the role of Ser473
17   phosphorylation in regulating PKBα activity. However,
18   although MAPKAP kinase-2 activity is stimulated to a
19   small extent by insulin in L6 cells, no activation
20   could be detected in 293 cells in response to insulin
21   or IGF-1. Moreover, exposure of L6 cells or 293 cells
22   to a chemical stress (0.5 mM sodium arsenite) strongly
23   activated MAPKAP kinase-2 (D. Alessi, unpublished work)
24   as found in other cells (Rouse et al, 1994; Cuenda et
25   al, 1995), but did not activate PKBα at all.
26   Furthermore, the drug SB 203580 which is a specific
27   inhibitor of the protein kinase positioned immediately
28   upstream of MAPKAP kinase-2 (Cuenda et al, 1995),
29   prevented the activation of MAPKAP kinase-2 by arsenite
30   but had no effect on the activation of PKBα by insulin
31   or IGF-1. Finally, the activation of PKBα was prevented
32   by wortmannin (Figs 6 and 9), but wortmannin had no
33   effect on the activation of MAPKAP kinase-2 in L6 or
34   293 cells. It should also be noted that the sequence
35   surrounding Ser-473 of PKBα (HFPQFSY) does not conform
36   to the optimal consensus for phosphorylation by MAPKAP

1   kinase-2 which requires Arg at position n-3 and a bulky
2   hydrophobic residue at position n-5, (where n is the
3   position of the phosphorylated residue). The Km for
4   phosphorylation of the synthetic peptide comprising
5   residues 465-478 is nearly 100-fold higher than the Km
6   for the standard MAPKAP kinase-2 substrate peptide
7   (data not shown). It is therefore unlikely that MAPKAP
8   kinase-2 mediates the phosphorylation of Ser-473 in
9   vivo.
10
11   The enzyme(s) which phosphorylates Thr-308 and Ser-473
12   in vivo does not appear to be PKBα itself. Thus
13   incubation of the partially active Asp-308 mutant with
14   MgATP did not result in the phosphorylation of Ser-473,
15   phosphorylation of the latter residue only occurring
16   when MAPKAP kinase-2 was added (Fig 11A, Fig 12).
17   Similarly, Thr-308 did not become phosphorylated when
18   either the partially active D473 mutant or the
19   partially active Ser-473 phosphorylated form of PKBα
20   were incubated with MgATP. PKBα when bound to lipid
21   vesicles containing phosphatidylserine and PIP3 also
22   fails to activate upon incubation with MgATP (Alessi et
23   al, 1996) and after transfection into 293 cells, a
24   "kinase dead" mutant of PKBα became phosphorylated on
25   Thr-308 and Ser-473 in response to insulin or IGF-1
26   (Fig 14). Furthermore, HA-PKBα from either unstimulated
27   or insulin-stimulated 293 cells failed to phosphorylate
28   the synthetic C-terminal peptide comprising amino acids
29   467-480.
30
31   In unstimulated L6 myotubes, the endogenous PKBα was
32   phosphorylated at a low level at a number of sites (Fig
33   6A), but in unstimulated 293 cells the transfected
34   enzyme was heavily phosphorylated at Ser-124 and
35   Thr-450 (Fig 10). Ser-124 and Thr-450 are both followed
36   by proline residues suggesting the involvement of

1   "proline-directed" protein kinases. Although, the

2   phosphorylation of Ser-124 was greatly decreased when

3   "kinase dead" PKBα was transfected into 293 cells (Fig

4   14), it would be surprising if Ser-124 is

5   phosphorylated by PKBα itself because the presence of a

6   C-terminal proline abolishes the phosphorylation of

7   synthetic peptides by PKBα (D.Alessi, unpublished

8   work). Since transfected PKBα is inactive in

9   unstimulated 293 cells (Fig 12), phosphorylation of

10   Ser-124 and Thr-450 clearly does not activate PKBα

11   directly. Ser-124 is located in the linker region

12   between the PH domain and the catalytic domain of the

13   mammalian PKBα isoforms but, unlike Thr-450, is not

14   conserved in the Drosophila homologue (Andjelkovic et

15   al, 1995).

16

17   While we do not wish to be bound by hypotheses, the

18   results described suggest that agonists which activate

19   PI 3-kinase are likely to stimulate PKBα activity via

20   one of the following mechanisms. Firstly, PIP3 or

21   PI3,4-bisP may activate one or more protein kinases

22   which then phosphorylate PKBα at Thr-308 and Ser-473.

23   Secondly, the formation of PIP3 may lead to the

24   recruitment of PKBα to the plasma membrane where it is

25   activated by a membrane-associated protein kinase(s).

26   The membrane associated Thr-308 and Ser-473 kinases

27   might themselves be activated by PIP3 and the

28   possibility that Thr-308 and/or Ser-473 are

29   phosphorylated directly by PI 3-kinase has also not

30   been excluded, because this enzyme is known to

31   phosphorylate itself (Dhand et al, 1994) and other

32   proteins (Lam et al, 1994) on serine residues.

33

34   **Example 8: Molecular basis for substrate specificity of**

35   **PKB.** PKBα has been shown to influence GSK3 activity.

36   GSK3α and GSK3β are phosphorylated at Ser-21 and Ser-9,

1   respectively, by two other insulin-stimulated protein

2   kinases, namely p70 S6 kinase and MAP kinase-activated

3   protein kinase-1 (MAPKAP-K1, also known as p90 S6

4   kinase). However, these enzymes are not rate-limiting

5   for the inhibition of GSK3 by insulin in L6 myotubes

6   because specific inhibitors of their activation

7   (rapamycin-p70 S6 kinase; PD 98059-MAPKAP kinase-1)

8   have no effect (Cross et al., 1995).

9

10   The activation of PI 3-kinase is essential for many of

11   the effects of insulin and growth factors, including

12   the stimulation of glucose transport, fatty acid

13   synthesis and DNA synthesis, protection of cells

14   against apoptosis and actin cytoskeletal rearrangements

15   (reviewed in Carpenter et al., 1996). These

16   observations raise the question of whether PKBα

17   mediates any of these events by phosphorylating other

18   proteins. To address this issue we characterised the

19   substrate specificity requirements of PKBα. We find

20   that the optimal consensus sequence for phosphorylation

21   by PKBα is the motif Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd,

22   where Yaa and Zaa are small amino acids (other than

23   glycine) and Hyd is a large hydrophobic residue (such

24   as Phe or Leu). We also demonstrate that PKBα

25   phosphorylates histone H2B (a substrate frequently used

26   to assay PKBα in vitro) at Ser-36 which lies in an Arg-

27   Xaa-Arg-Xaa-Xaa-Ser-Hyd motif. These studies identified

28   a further PKBα substrate (Arg-Pro-Arg-Ala-Ala-Thr-Phe)

29   that, unlike other peptides, is not phosphorylated to a

30   significant extent by either p70 S6 kinase or MAPKAP-

31   K1.

32

33

34   **Results**

35   **Preparation of Protein Kinase Bα**

36   In order to examine the substrate specificity of PKBα,

1   it was first necessary to obtain a kinase preparation

2   that was not contaminated with any other protein kinase

3   activities. 293 cells were therefore transiently

4   transfected with a DNA construct expressing

5   haemagglutonin-tagged PKBα (HA-PKBα), stimulated with

6   IGF-1 and the HA-PKBα immunoprecipitated from the

7   lysates). IGF-1 stimulation resulted in a 38-fold

8   activation of PKBα (Fig 16) and analysis of the

9   immunoprecipitates by SDS-polyacrylamide gel

10 electrophoresis revealed that the 60 kDa PKBα was the

11 major protein staining with Coomassie Blue apart from

12 the heavy and light chains of the haemagglutonin

13 monoclonal antibody (Fig 16, Lanes 2 and 3). The minor

14 contaminants were present in control immunoprecipitates

15 derived from 293 cells transfected with an empty pCMV5

16 vector but lacked HA-PKB activity (Fig 16, Lane 4).

17 Furthermore, a catalytically inactive mutant HA-

18 PKBα_immunoprecipitated from the lysates of IGF-1

19 stimulated 293 cells had no Crosstide kinase activity

20 (Alessi et al., 1996). Thus, all the Crosstide activity

21 in HA-PKB immunoprecipitates is catalysed by PKBα.

22

23 **Identification of the residues in histone H2B**

24 **phosphorylated by PKBα.** Currently, three substrates are

25 used to assay PKBα activity in different laboratories,

26 histone H2B, MBP and Crosstide. PKBα phosphorylated

27 Crosstide with a Km of 4 μM and a Vmax of 260 U/mg

28 (Table 7.1 A, peptide 1), histone H2B with a Km of 5 μM

29 and a Vmax of 68 U/mg, and MBP with a Km of 5 μM and a

30 Vmax of 25 U/mg. Thus the Vmax of histone H2B and MBP

31 are 4-fold and 10-fold lower than for Crosstide. In

32 order to identify the residue(s) in histone H2B

33 phosphorylated by PKBα, ^{32}P-labelled histone H2B was

34 digested with trypsin (see Methods) and the resulting

35 peptides chromatographed on a C18 column at pH 1.9.

36 Only one major ^{32}P-labelled peptide (termed T1) eluting

1   at 19.5 % acetonitrile was observed (Fig 17A), The

2   peptide contained phosphoserine (data not shown), its

3   sequence commenced at residue 34 of histone H2B and a

4   single burst of radioactivity occurred after the third

5   cycle of Edman degradation (Fig 17B), demonstrating

6   that PKBα phosphorylates histone H2B at Ser-36 within

7   the sequence Arg-Ser-Arg-Lys-Glu-Ser-Tyr. Thus, like

8   the serine phosphorylated in Crosstide, Ser-36 of

9   histone H2B lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd

10   motif (where Hyd is a bulky hydrophobic residue -Phe in

11   Crosstide, Tyr in H2B).

12

13   **Molecular basis for the substrate specificity of PKBα**

14   To further characterise the substrate specificity

15   requirements for PKBα, we first determined the minimum

16   sequence phosphorylated efficiently by PKBα by removing

17   residues sequently from the C-terminal and N-terminal

18   end of Crosstide. Removal of the N-terminal glycine and

19   up to three residues from the C-terminus had little

20   effect on the kinetics of phosphorylation by PKBα

21   (Table 7.1A, compare peptides 1 and 5). However any

22   further truncation of either the N or C-terminus

23   virtually abolished phosphorylation (Table 7.1A,

24   peptides 8 and 9). The minimum peptide phosphorylated

25   efficiently by PKBα (Arg-Pro-Arg-Thr-Ser-Ser-Phe) was

26   found to be phosphorylated exclusively at the second

27   serine residue as expected. Consistent with this

28   finding, a peptide in which this serine was changed to

29   alanine was not phosphorylated by PKBα (Table 7.1A,

30   peptide 7). All further studies were therefore carried

31   out using variants of peptide 5 in Table 7.1A (see

32   below).

33

34   A peptide in which the second serine of peptide 5

35   (Table 7.1A) was replaced by threonine was

36   phosphorylated with a Km of 30 μM and an unchanged Vmax

1   (Table 7.1, peptide 6). All the ^{32}P-radioactivity

2   incorporated was present as phosphothreonine and solid

3   phase sequencing revealed that the peptide was only

4   phosphorylated at the second threonine residue, as

5   expected (data not shown). Thus PKBα is capable of

6   phosphorylating threonine as well as serine residues,

7   but has a preference for serine.

8

9   We next changed either of the two arginine residues in

10   peptide 5 to lysine. These substitutions drastically

11   decreased the rate of phosphorylation by PKBα (Table

12   7.1A, peptides 10 and 11) demonstrating a requirement

13   for arginine (and not simply any basic residue) at both

14   positions.

15

16   We then examined the effect of substituting the

17   residues situated immediately C-terminal to the

18   phosphorylated serine in peptide 5 (Table 7.1B). The

19   data clearly demonstrate that the presence of a large

20   hydrophobic residue at this position is critical for

21   efficient phosphorylation, with the Km increasing

22   progressively with decreasing hydrophobicity of the

23   residue at this position (Table 7.1B, peptides 1 to 4).

24   Replacement of the C-terminal residue with Lys

25   increased the Km 18-fold and a substitution at this

26   position with either Glu or Pro almost abolished

27   phosphorylation (Table 7.1B, peptides 5-7).

28

29   Replacement of the Thr situated two residues N-terminal

30   to the phosphorylated serine increased the Km with any

31   amino acid tested (Table 7.1C). Substitution with Ala

32   only increased Km by 2-3 fold, but substitution with

33   other residues was more deleterious and with Asn (a

34   residue of similar size and hydrophilicity to Thr)

35   phosphorylation was almost abolished (Table 7.1C).

36   Replacement of the Ser situated one residue N-terminal

1   to the phosphorylated serine also increased the Km with

2   any amino acid tested, but the effects were less severe

3   than at position n-2 (Table 7.1C). When residues n-2

4   and n-1 were both changed to Ala, the resulting peptide

5   RPRAASF was phosphorylated by PKBα with a Km only 5-

6   fold higher than RPRTSSF. In contrast the peptides

7   RPRGGSF, RPRAGSF, and RPRGASF were phosphorylated less

8   efficiently (Table 7.1C).

9

10 **Comparison of the substrate specificity of PKBα with**

11 **MAPKAP kinase-1, and p70 S6 kinase.** Since MAPKAP-K1

12 and p70 S6 kinase phosphorylate the same residue in

13 GSK3 phosphorylated by PKBα, and studies with synthetic

14 peptides have established that MAPKAP-K1 and p70 S6

15 kinase also preferentially phosphorylate peptides in

16 which basic residues are present at positions n-3 and

17 n-5 (Leighton et al., 1995), we compared the

18 specificities of MAPKAP-K1, p70 S6 kinase and PKBα in

19 greater detail.

20

21 MAPKAP kinase-1 and p70 S6 kinase phosphorylate the

22 peptides KKKNRTLSVA and KKRNRTLSVA with extremely low

23 Km values of 0.2- 3.3 μM, respectively (Table 7.2).

24 However, these peptides were phosphorylated by PKBα

25 with 50-900 fold higher Km values (Table 7.2A, peptides

26 1 and 2). The peptide KKRNRTLTV, which is a relatively

27 specific substrate for p70 S6 kinase (Leighton et al.,

28 1995) was also phosphorylated very poorly by PKBα

29 (Table 7.2A, peptide 4).

30

31 Crosstide is phosphorylated by p70 S6 kinase and MAPKAP

32 kinase-1 with similar efficiency to PKBα ((Leighton et

33 al., 1995); Table 7.2B-peptide 1 and Fig 18). However,

34 truncation of Crosstide to generate the peptide RPRTSSF

35 was deleterious for phosphorylation by MAPKAP-K1 and

36 even worse for p70 S6 kinase (Table 7.2B-peptides 1 and

1   2 and Fig 18). Moreover, changing the phosphorylated
2   serine in RPRTSSF to threonine increased the Km for
3   phosphorylation by p70 S6 kinase much more than for
4   PKBα and almost abolished phosphorylation by MAPKAP-K1
5   (Table 7.2B-peptide 3 and Fig 18). The peptide RPRAASF
6   was phosphorylated by MAPKAP-K1 with essentially
7   identical kinetics to that of PKBα; however
8   phosphorylation by p70 S6 kinase was virtually
9   abolished (Table 7.2B-peptide 4 and Fig 18). Based on
10 these observations we synthesized the peptide RPRAATF.
11 This peptide was phosphorylated by PKBα with a Km of
12 25μM and similar Vmax to RPRTSSF, but was not
13 phosphorylated to a significant extent by either
14 MAPKAP-K1 or p70 S6 kinase (Table 7.2B-peptide 5, Fig
15 18). In Fig 18, the protein kinase concentration in
16 the assays towards Crosstide were 0.2 U/ml, and each
17 peptide substrate was assayed at a concentration of 30
18 μM. Filled bars denote PKBα activity, hatched bars
19 MAPKAP kinase-1 activity, and grey bars p70 S6 kinase
20 activity. The activities of each protein kinase are
21 given relative to their activity towards Crosstide
22 (100). The results are shown ± SEM for two experiments
23 each carried out in triplicate.
24
25 ## Discussion.
26 We have established that the minimum consensus sequence
27 for efficient phosphorylation by PKBα is Arg-Xaa-Arg-
28 Yaa-Zaa-Ser-Hyd, where Xaa is any amino acid, Yaa and
29 Zaa are small amino acid other than glycine (Ser, Thr,
30 Ala) and Hyd is a bulky hydrophobic residue (Phe, Leu)
31 (Table 7.1). The heptapeptide with the lowest Km value
32 was RPRTSSF, its Km of 5 μM being comparable to many of
33 the best peptide substrates identified for other
34 protein kinases. The Vmax for this peptide (250 nmoles
35 min-1 mg-1) may be an underestimate because the PKBα
36 was obtained by immunoprecipitation from extracts of

43

1   IGF-1 stimulated 293 cells over-expressing this protein
2   kinase, and a significant proportion of the PKBα may
3   not have been activated by IGF-1 treatment.
4
5   The requirement for arginine residues at positions n-3
6   and n-5 (where n is the site of phosphorylation) seems
7   important, because substituting either residue with
8   lysine decreases phosphorylation drastically. Serine
9   and threonine residues were preferred at positions n-1
10 and n-2, although the Km value was only increased about
11 5-fold if both of these residues were changed to Ala.
12 Serine was preferred at position n, since changing it
13 to threonine caused a six-fold increase in the Km.
14 The peptide RPRAATF, which was phosphorylated with a Km
15 of 25 μM and similar Vmax to RPRTSSF, may therefore be
16 a better substrate for assaying PKBα in partially
17 purified preparations, because unlike Crosstide, it
18 contains only one phosphorylatable residue and is not
19 phosphorylated significantly by MAPKAP-K1 or p70 S6
20 kinase (Table 7.2, Fig 18 and see below).
21
22 The Proline at position n-4 was not altered in this
23 study because it was already clear that this residue
24 was not critical for the specificity of PKBα. Residue
25 n-4 is proline in GSK3β but alanine in GSK3α. Both GSK3
26 isoforms are equally good substrates for PKBα in vitro
27 (Cross et al., 1995), and the peptide
28 GRARTSSFA (corresponding to the sequence in GSK3α) is
29 phosphorylated by PKBα with a Km of 10 μM and Vmax of
30 230 U/mg (Table 7.1A, peptide 2). Moreover, in histone
31 H2B, the residue located four amino acids N-terminal to
32 the PKBα phosphorylation site is serine (Fig 17). The
33 presence of Glu and Lys at positions n-1 and n-2 may
34 explain why histone H2B is phosphorylated by PKBα with
35 a four-fold lower Vmax than the peptide RPRTSSF.
36

1   Two other protein kinases which are activated by

2   insulin and other growth factors, p70 S6 kinase and

3   MAPKAP-K1, require basic residues at positions n-3 and

4   n-5 (Leighton et al., 1995), explaining why they also

5   phosphorylate and inactivate GSK3 in vitro (Sutherland

6   et al., 1993). Indeed, there is evidence that MAPKAP-

7   K1 plays a role in the inhibition of GSK3 by EGF

8   because, unlike inhibition by insulin which is

9   prevented by inhibitors of PI 3-kinase, the inhibition

10   of GSK3 by EGF is only suppressed partially by

11   inhibitors of PI 3-kinase. Moreover, in NIH 3T3 cells,

12   the inhibition of GSK3α and GSK3β by EGF is largely

13   prevented by expression of a dominant negative mutant

14   of MAP kinase kinase-1 (Eldar et al., 1995). In

15   contrast, p70 S6 kinase is not rate limiting for the

16   inhibition of GSK3 in the cells that have been examined

17   so far because rapamycin, which prevents the activation

18   of p70 S6 kinase by EGF or insulin, has no effect on

19   the inhibition of GSK3 by these agonists (Cross et al.,

20   1995 and Saito et al., 1994).

21

22   Additional similarities between p70 S6 kinase, MAPKAP-

23   K1 and PKBα include the failure to phosphorylate

24   peptides containing Pro at position n+1 and dislike of

25   a lysine at the same position. This suggests that, in

26   vivo, these kinases are unlikely to phosphorylate the

27   same residues as MAP kinases (which phosphorylates

28   Ser/Thr-Pro motifs) or protein kinase C (which prefers

29   basic residues C-terminal to the site of

30   phosphorylation). However, the present work has also

31   revealed significant differences in the specificities

32   of these enzymes. In particular MAPKAP-K1 and (to a

33   lesser extent) p70 S6 kinase can tolerate substitution

34   of the Arg at position n-5 by lysine whereas PKBα

35   cannot (see Table 7.1A, Table 7.2A and (Leighton et

36   al., 1995)). MAPKAP-K1 and p70 S6 kinase can also

1   tolerate, to some extent, substitution of Arg at
2   position n-3 by Lys.   For example, the peptide
3   KKRNKTLSVA is phosphorylated by MAPKAP-K1 and p70 S6
4   kinase with Km values of 17 and 34 µM, respectively,
5   as compared to Km values of 0.7 and 1.5 µM for the
6   peptide KKRNRTLSVA (Table 7.2A). In contrast, PKBα
7   does not phosphorylate the peptide KKRNKTLSVA (Table
8   7.2A) or any other peptide that lacks Arg at position
9   n-3. PKBα and p70 S6 kinase, but not MAPKAP-K1,
10 phosphorylate Thr as well as Ser (Table 7.1A) and can
11 phosphorylate peptides lacking any residue at position
12 n+2 ((Leighton et al., 1995) and Table 7.2A), while
13 PKBα and MAPKAP-K1, but not p70 S6 kinase, can tolerate
14 substitution of both the n-1 and n-2 positions of the
15 peptide RPRTSSF with Ala (Table 7.2B). These
16 differences explain why the peptide RPRAATF is a
17 relatively specific substrate for PKBα.
18
19 One of the best peptide substrates for MAPKAP-K1 and
20 p70 S6 kinase (KKRNRTLSVA) was a poor substrate for
21 PKBα (Table 7.2, peptide 2), despite the presence of
22 Arg at positions n-3 and n-5. The presence of Leu at
23 position n-1 and Val at position n+1 are likely to
24 explain the high Km for phosphorylation, because PKBα
25 prefers a small hydrophilic residue at the former
26 position and a larger hydrophobic residue at the latter
27 position (Tables 7.1 and 7.2).
28

29 **Example 9:**
30 This example demonstrates that coexpression of GSK3 in
31 293 cells with either the wild type or a constitutively
32 activated PKB results in GSK3 becoming phosphorylated
33 and inactivated. However coexpression of a mutant of
34 GSK3 in which Ser-9 is mutated to an Ala residue is not
35 inactivated under these conditions. These experiments
36 provide further evidence that PKBa activation can

1 mediate the phosphorylation and inactivation of GSK3 in
2 a cellular environment, and could be used as an assay
3 system to search for specific PKB inhibitors.
4
5 Monoclonal antibodies recognising the sequence EFMPME
6 (EE) antibodies and the (EQKLISEEDL) c-Myc purchased
7 from Boehringer (Lewis, UK).
8
9 **Construction of expression vectors and transfections**
10 **into 293 cells.** HA-PKBa, HA-KD-PKB and 308D/473D
11 HA-PKBa was described previously (Alessi et al.. 1996).
12
13 A DNA construct expressing human GSK3B with the EFMPME
14 (EE) epitope tag at the N-terminus was prepared as
15 follows: A standard PCR reaction was carried out using
16 as a template the human GSK3β cDNA clone in the
17 pBluescript SK+ vector and the oligonucleotides
18
19 GCGG<u>AGATCT</u>GCCACCATGGAGTTCATGCCCATGGAGTCAGGGCGGCCCAGAACC
20
21 and GCGG<u>TCCGGA</u>ACATAGTCCAGCACCAG that incorporate a *bgl*
22 II site (underlined) and a *Bspe I* site (double
23 underlined). A three-way ligation was then set up in
24 which the resulting PCR product was subcloned as a *Bgl*
25 *II-Bspe* I fragment together with the C-terminal Bspe
26 I-Cla I fragment of GSK3β into the Bgl II-Cla I sites
27 of the pCMV5 vector (Anderson et al., 1989). The
28 construct was verified by DNA sequencing and purified
29 using the Quiagen plasmid Mega kit according to the
30 manufacturers protocol. The c-Myc GSK3, BA9 construct
31 encodes GSK3β in which Ser-9 is mutated to Ala and
32 possesses a c-myc epitope tag at the C-terminus and was
33 prepared as described in Sperber et al., 1995. The
34 c-Myc GSK3β A9 gene was then subcloned into xba I/ECOR
35 I sites of the pCMV5 eukaryotic expression vector.
36

1   **Cotransfection of GSK3β with PKBa and its assay.**

2   293 cells growing on 10 cm diameter dishes were

3   transfected with 10 ug of DNA constructs expressing

4   EE-GSK3, Myc-GSK3A9 in the presence or absence of

5   HA-PKB, HA-KD-PKB or HA-308D/473D-PKB exactly as

6   described in Alessi et al., 1996. The cells were grown

7   in the absence of serum for 16 h prior to lysis, and

8   then lysed in 1.0 ml of ice-cold Buffer A (50 mM

9   Tris/HCI pH 7.5,1 mM EDTA 1 mM EGTA, 1% (by vol) Triton

10   X100, 1 mM sodium orthopervanadate, 10 mM sodium

11   glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate,

12   1uM Microcystin-LR, 0.27 M sucrose, 1 mM benzamidine,

13   0.2 mM phenylmethylsulphonyl fluoride, 10 ug/ml

14   leupeptin, and 0.1% (by vol) 2-mercaptoethanol). The

15   lysate was centrifuged at 4°C for 10 min at 13, 000 x g

16   and an aliquot of the supernatant (100 ug protein) was

17   incubated for 30 min on a shaking platform with 5 ul of

18   protein G-Sepharose coupled to 1ug of EE monoclonal

19   antibody. The suspension was centrifuged for 1min at

20   13,000 x g, the Protein G-Sepharose-antibody-EE-GSK3β

21   complex washed twice with 1.0 ml of Buffer A containing

22   0.5 M NaCl, and three times with Buffer B (50 mM Tris

23   pH 7.5, 0.1 mM EGTA, 0.01% (by vol) Brij-35 and 0.1%

24   (by vol) 2-mercaptoethanol), and the immunoprecipitate

25   assayed for GSK3 activity after incubation with either

26   PP2A or microcystin inactivated PP2A as described

27   previously (Cross et al., 1994).

28

29   **Results**

30

31   **Cotransfection of GSK3β with PKBa in 293 cells results**

32   **in GSK3 phosphorylation and inactivation**

33   Human embryonic kidney 293 cells were transfected with

34   a DNA construct expressing EE-epitope tagged GSK3β

35   either in the presence or absence of DNA constructs

36   expressing wild type-PKBa, a catalytically inactive

1 PKBa or a constitutively active HA-(308D/473D)-PKBa.
2 Cells were serum starved for 16 h. 36h post
3 transfection the cells were lysed, and the GSK3β
4 immunoprecipitated from the lysates using monoclonal EE
5 antibodies and the GSK3β activity measured before and
6 after treatment with PP2A. When EEGSK3β was expressed
7 alone or in the presence of a catalytically inactive
8 PKBa, treatment of the EE-GSK3β with PP2A only resulted
9 in about a 12% increase in activity (Fig 19A). However
10 when EE-GSK3β was coexpressed with either the wild type
11 PKBa or the constitutively activated 308D/473D-HA-PKBa,
12 treatment of the EE-GSK3 from these cell lysates with
13 PP2A resulted in a 68% and 85% increase in the GSK3
14 activity, respectively. Coexpression of Myc-GSK3β A9
15 with HA-PKB or the constitutively active
16 308D/473D-HA-PKBa did not result in any significant
17 inactivation of this mutant of GSK3 as judged by its
18 ability to be reactivated by PP2A (Fig 19B). These data
19 demonstrate that even in a cellular environment, PKBa
20 is capable of phosphorylating GSK3β at Ser-9 and
21 inactivation of the enzyme. To estimate the relative
22 levels of EE-GSK3β and PKBa, EE-GSK3 and HA-PKBa were
23 immunoprecipitated from equal volumes of cell lysate,
24 and the immunoprecipitates run on an SDS-polyacrylamide
25 gel, and the gel stained with Coomassie Blue. These
26 experiments revealed that both the wild type HA-PKBa
27 and the 308D/473D-PKBa were expressed at a 20 to 30
28 -fold higher level than GSK3a, whereas KD-PKBa is
29 expressed at a level that is about 5-fold lower than
30 that of the wild type PKBa. Under the conditions used
31 for the immunoprecipitations, no PKBa was
32 co-immnuoprecipitated with GSK3β, or no GSK3β was
33 co-immunoprecipitated with the PKBa (data not shown).
34 Coexpression of EE-GSK3β with all forms of PKBa
35 resulted in about a 2-3 fold decrease in the level of
36 expression on EE-GSK3β compared to when it is expressed

1   alone in cells.

2

3   **Example 10: basic assay for identifying agents which**

4   **affect the activity of PKB.**

5   A 40 μl assay mix was prepared containing protein

6   kinase (0.2U/ml) in 50 mM Tris/HCI pH 7.5, 0.1 mM EGTA,

7   0.1% (by vol) 2-mercaptoethanol, 2.5 μM PKI, protein

8   kinase substrate (30μM), and the indicated

9   concentration of Ro-318220 or GC 109203X (test

10   inhibitors). After incubation on ice for 10 min the

11   reaction was started by the addition of 10 μl of 50mM

12   magnesium acetate and 0.5 mM [γ^{32}P]ATP (100-200

13   cpm/pmol). For the assay of mixed isoforms of PKC 20

14   μM diacylglycerol, 0.5 mM CaCl$_2$, and 100 μM

15   phosphatidylserine were also present in the

16   incubations. The assays were carried out for 15 min at

17   30°C, then terminated and analysed as described (Alessi

18   1995). One unit of activity was that amount of enzyme

19   that catalysed the phosphorylation of 1nmol os

20   substrate in 1 min. The final concentration of DMSo in

21   each assay was 1% (by vol). This concentration of DMSO

22   does not inhibit any of these enzymes. Mixed isoforms

23   of PKC were assayed using histone H1 as substrate,

24   while MAPKAP-K1β and p70 S6 kinase were assayed using

25   the peptide KKRNRTLSVA (Leighton 1995). Protein kinase

26   B was assayed with the peptide GRPRTSSFAEG [9] and

27   MAPKAP-K2 was assayed with the peptide KKLNRTLSVA

28   (Stokoe 1993). p42 MAP kinase was assayed using MBP,

29   and MAPKK-1, and c-Raf1 were assayed as described in

30   Alessi 1995.

31

32   **Results**

33   Effect of Ro 318220 and GF 109203X on·protein kinases

34   activated by growth factors, cytokines and cellular

35   stresses. The mixed isoforms of PKC were potently

36   inhibited by Ro 318220, with an IC$_{50}$ of 5 nM in our

1   assay (Fig 20A). In contrast, a number of protein

2   kinases activated by growth factors (c-Raf1, MAPKK-1,

3   p42 MAP kinase) and one protein kinase that is

4   activated by cellular stresses and proinflammatory

5   cytokines (MAPKAP-K2) were not inhibited significantly

6   by Ro 318022 in vitro (Fig 20A). Protein kinase B, an

7   enzyme that is activated in response to insulin and·

8   growth factors was inhibited by Ro 318220 (IC_{50} of 1 μM,

9   Fig 20B) similar to the IC_{50} for PKA. However, to our

10   surprise, MAPKAP-K1B an enzyme which lies immediately

11   downstream of p42 and p44 MAP kinases and which is

12   activated in response to every agonist that stimulates

13   this pathway, was inhibited by Ro 318220 even more

14   potently than the mixed PKC isoforms (IC_{50} = 3nm, Fig

15   20B). The p70 S6 kinase, which lies on a distinct

16   growth factor-stimulated signalling pathway from

17   MAPKAP-K1B, was also potently inhibited by Ro 318220

18   (IC_{50}=15 nM, Fig 20B).

19

20   Similar results were obtained using GF 109203X instead

21   of Ro 3318220. As reported previously (Toullec et al.,

22   1991), GC 109203X inhibited the mixed isoforms of PKC

23   (IC_{50}=30 nM) without inhibiting protein kinase B (Fig

24   21) or c-Raf, MAPKK-1 and p42 MAP kinase (data not

25   shown). However MAPKAP-K1B and p70 S6 kinase were

26   potently inhibited by this compound with IC_{50} values of

27   50 nM and 100 nM, respectively (Fig 21).

28

29

51

1	**General Materials and Methods** Tissue culture reagents, myelin
2	basic protein (MBP), microcystin-LR, and IGF-1 were obtained from
3	Life Technologies Inc. (Paisley, UK), insulin from Novo-Nordisk
4	(Bagsvaerd, Denmark), phosphate free Dulbecco's minimal essential
5	medium (DMEM) from (ICN, Oxon, UK), Protein G-Sepharose and
6	CH-Sepharose from Pharmacia (Milton Keynes, UK), alkylated trypsin
7	from Promega (Southampton, UK), 4-vinylpyridine, wortmannin and
8	fluroisothiocyanante-labelled antimouse IgG from goat from
9	Sigma-Aldrich (Poole, Dorset, UK). Polyclonal antibodies were
10	raised in sheep against the peptides RPHFPQFSYSASGTA
11	(corresponding to the last 15 residues of rodent PKBα) and
12	MTSALATMRVDYEQIK (corresponding to residues 352 to 367 of human
13	MAPKAPkinase-2) and affinity purified on peptide-CH-Sepharose.
14	Monoclonal HA antibodies were purified from the tissue culture
15	medium of 12CA5 hybridoma and purified by chromatography on
16	Protein GSepharose. The peptide RPRHFPQFSYSAS, corresponding to
17	residues 465-478 of PKBα, was synthesized on an Applied Biosystems
18	430A peptide synthesizer. cDNA encoding residues 46-400 of human
19	MAPKAP kinase-2 was expressed in E.coli as a glutathione
20	S-transferase fusion protein and activated with p38/RK MAP KINASE
21	by Mr A.Clifton (University of Dundee) as described previously
22	(Ben-Levy et al., 1995).
23	
24	Monoclonal antibodies recognising the haemagglutonin (HA) epitope
25	sequence YPYDVPDYA, Protein G-Sepharose and histone H2B were
26	obtained from Boehringer (Lewes, UK). MAPKAP kinase-1 (Sutherland
27	et al., 1993) and p70 S6 kinases (Leighton et al., 1995) were
28	purified from rabbit skeletal muscle and rat liver respectively.
29	
30	**Construction of expression vectors.** The pECE constructs encoding
31	the human HAPKBα and kinase-dead (K179A) HA-KD-PKBα have already
32	been described (Andjelkovic at al., 1996). The mutants at Ser-473
33	(HA-473A PKBα and HA-473D PKBα were created by PCR using a 5′
34	oligonucleotide encoding amino acids 406 - 414 and mutating 3′
35	oligonucleotide encoding amino acids 468 - 480, and the resulting
36	PCR products subcloned as *CelII-EcoRI* fragment into pECE.HA-PKBα.
37	The Thr-308 mutants (HA-308A PKBα and HA308D PKBα) were created by
38	the two-stage PCR technique (No et al., 1989) and subcloned as
39	*NotI-EcoRI* fragments into pECE.HA-PKB. The double mutant
40	HA-308D/473D PKB was made by subcloning the CelII-EcoRI fragment
41	encoding 473D into pECE.HA-308D PKBα. For construction of
42	cytomegalovirus-driven expression constructs, *BglII-XbaI* fragments
43	from the appropriate pECE constructs were subcloned into the same
44	restriction sites of the pCMV5 vector (Andersson et al., 1989).

1   All constructs were confirmed by restriction analysis and
2   sequencing and purified using Quiagen Plasmid Maxi Kit according
3   to the manufacturer's protocol. All oligonucleotide sequences are
4   available upon request.
5
6   ^{32}P-labelling of L6 myotubes and immunoprecipitation of PKBα. L6
7   cells were differentiated into myotubes on 10 cm diameter dishes
8   (Hundal et al., 1992). The myotubes were deprived of serum
9   overnight in DMEM, washed three times in phosphate free DMEM and
10   incubated for a further 1 h with 5 ml of this medium. The myotubes
11   were then washed twice with phosphate free DMEM and incubated for
12   4 h with carrier-free [^{32}P]orthophosphate (1 mCi/ml). Following
13   incubation in the presence or absence of 100 nM wortmannin for 10
14   min, the myotubes were stimulated for 5 min at 37°c in the
15   presence or absence of 100 nM insulin and placed on ice, The
16   medium was aspirated,the myotubes washed twice with ice-cold DMEM
17   buffer and then lysed with 1.0 ml of ice-cold Buffer A (50 mM
18   Tris/HCl pH 7.5,1 mM EDTA 1 mM EGTA, 1% (by vol) Triton X100, 1 mM
19   sodium orthopervanadate, 10 mM sodium glycerophosphate, 50 mM NaF,
20   5 mM sodium pyrophosphate, 1 μM Microcystin-LR, 0.27 M sucrose, 1
21   mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 10 μg/ml
22   leupeptin, and 0.1% (by vol) 2-mercaptoethanol). The lysates were
23   centrifuged at 4°C for 10 min at 13,000 x g and the supernatants
24   incubated for 30 min on a shaking platform with 50 μl of Protein
25   G-Sepharose coupled to 50 μg of preimmune sheep IgG. The
26   suspensions were centrifuged for 2 min at 13,000 x g and the
27   supernatants incubated for 60 min with 30 μl of Protein G--
28   Sepharose covalently coupled to 60 μg of PKBα antibody (Harlow and
29   Lane, 1988). The Protein G-Sepharose-antibody-PKBα complex was
30   washed eight times with 1.0 ml of Buffer A containing 0.5 M NaCl,
31   and twice with 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA and 0.1% (by
32   vol) 2-mercaptoethanol (Buffer B).
33
34   Assay of immunoprecipitated PKBα and protein determinations. Three
35   aliquots of each immunoprecipitate (each comprising only 5% of the
36   total immunoprecipitated PKBα) were assayed for PKBα activity
37   towards the peptide GRPRTSSFAEG as described previously (Cross et
38   al., 1995). One unit of activity was that amount which catalysed
39   the phosphorylation of 1 nmol of substrate in 1 min. Protein
40   concentrations were determined by the method of Bradford, 1976.
41
42   Tryptic digestion of in vivo phosphorylated PKBα. The
43   immunoprecipitated PKBα was added to an equal volume of 2% (by

53

mass) SDS and 2 % (by vol) 2-mercaptoethanol, and incubated for 5

min at 100°C, After cooling to room temperature, 4-vinylpyridine

was added to a final concentration of 2 % (by vol) and the mixture

was incubated for 1h at 30°C on a shaking platform, followed by

electrophoresis on a 10% polyacrylamide gel. After

autoradiography, the 60 kDa band corresponding to rat PKBα was

excised and the gel piece homogenized in five vols of 25 mM

N-ethylmorpholine HCl, pH 7.7, containing 0.1% (by mass) SDS and 5

% (by vol) 2-mercaptoethanol. The suspension was incubated for 1 h

at 37°C on a shaking platform, then centrifuged for 1 min at

13,000 x g and the supernatant collected. The pellet was incubated

for a further 1h with five vols of the same buffer and centrifuged

for 1min at 13,000 xg. The two supernatants (containing 80-90% of

the ^{32}P-radioactivity) were combined, 0.2 vols of 100% (by mass)

trichloroacetic acid added, and the sample incubated for 1 h on

ice. The suspension was centrifuged for 10 min at 13,000 x g, the

supernatant discarded and the pellet washed five times with 0.2 ml

of water. The pellet was then incubated at 30°C with 0.3 ml of 50

mM Tris/HCl pH 8.0, 0.1% (by vol) Triton X100 containing 1μg of

alkylated trypsin. After 3 h another 1μg of trypsin was added and

the suspension left for a further 12 h. Guanidinium hydrochloride

(8 M) was added to bring the final concentration to 1.0 M in order

to precipitate any residual SDS and, after standing on ice for 10

min, the suspension was centrifuged for 5 min at 13, 000 x g. The

supernatant containing 90 % of the ^{32}P-radioactivity was

chromatographed on a Vydac C18 column as described in the legend

to Fig 2.

Transfection of 293 cells and immunoprecipitation of HA-tagged

PKBα.   Human embryonic kidney 293 cells were cultured at 37°C in

an atmosphere of 5% CO2, on 10 cm diameter dishes in DMEM

containing 10 % foetal calf serum. Cells were split to a density

of 2 x 106 per 10 cm dish, and after 24 h at 37°C the medium was

aspirated and 10 ml of freshly prepared DMEM containing 10 %

foetal calf serum added. Cells were transfected by a modified

calcium phosphate method (Chen and Okayama, 1988) with 1ug/ml DNA

per plate. 10 μg of plasmid DNA in 0.45 ml of sterile water was

added to 50 μl of sterile 2.5 M CaCl2, and then 0.5 ml of a

sterile buffer composed of 50 mM N,N-bis[2-hydroxyethyl]-2-

aminoethanesulphonic acid/HCl pH 6.96, 0.28 M NaCl and 1.5 mM

Na2HPO4 was added. The resulting mixture was vortexed for 1 min,

allowed to stand at room temperature for 20 min, and then added

dropwise to a 10 cm dish of 293 cells). The cells were placed in

1   an atmosphere of 3% CO2, for 16 h at 37°C, then the medium was
2   aspirated, and replaced with fresh DMEM containing 10% foetal calf
3   serum. The cells were incubated for 12 h at 37°C in an atmosphere
4   of 5% CO2,, and then for 12 h in DMEM in the absence of serum.
5   Cells were preincubated for 10 min in the presence of 0.1% DMSO or
6   100 nM wortmannin in 0.1% DMSO and then stimulated for 10 min with
7   either 100 nM insulin or 50 ng/ml IGF-1 in the continued presence
8   of wortmannin. After washing twice with ice cold DMEM the cells
9   were lysed in 1.0 ml of icecold Buffer A, the lysate was
10 centrifuged at 4°C for 10 min at 13,000 x g and an aliquot of the
11 supernatant (10 µg protein) was incubated for 60 min on a shaking
12 platform with 5 µl of protein G-Sepharose coupled to 2 µg of HA
13 monoclonal antibody. The suspension was centrifuged for 1 min at
14 13,000 x g, the Protein G-Sepharose-antibody-HA-PKBα complex
15 washed twice with 1.0 ml of Buffer A containing 0.5 M NaCl, and
16 twice with Buffer B, and the immunoprecipitate assayed for PKBα
17 activity as described above.
18
19 ^{32}P-Labelling of 293 cells transfected with HA-PKBα. 293 cells
20 transfected with HA-PKBα DNA constructs. were washed with
21 phosphate free DMEM, incubated with [32p] orthophosphate (1
22 mCi/ml) as described for L6 myotubes, then stimulated with insulin
23 or IGF1 and lysed, and PKBα immunoprecipitated as described above.
24 The ^{32}P-labelled HA-PKBα immunoprecipitates were washed, alkylated
25 with 4-vinylpyridine, electrophoresed and digested with trypsin as
26 described above for the endogenous PKBα present in rat L6
27 myotubes.
28
29 Transfection of COS-1 cells and immunoprecipitation of HA-PKBα.
30 COS-1 cells were maintained in DMEM supplemented with 10% FCS at
31 370C in an atmosphere of 5% CO2,. Cells at 70 - 80% confluency
32 were transfected by a DEAE-dextran method (Seed & Aruffo, 1987),
33 and 48 hours later serum-starved for 24 hours. Cells were lysed in
34 a buffer containing 50 mM Tris-HCl, pH 7.5,120 mM NaCl, 1% Nonidet
35 P-40, 25 mM NaF, 40 mM sodium-,β-glycerophosphate, 0.1 mM sodium
36 orthopervanadate, 1 mM EDTA, 1mM benzamidine, 1 mM
37 phenylmethylsulphonyl fluoride, and lysates centrifuged for 15 min
38 at 13,000 x g at 4°C. Supernatants were pre cleared once for 30
39 min at 4°C with 0.1 vols of 50% Sepharose 4B/25% Pansorbin
40 (Pharmacia and Calbiochem, respectively) and HA-PKBα
41 immunoprecipitated from 1 mg of extract using the 12CA5 antibody
42 coupled to Protein A Sepharose beads. Immunoprecipitates were
43 washed twice with lysis buffer containing 0.5 M NaCl and once with

1   lysis buffer.

2

3   **Immunoblotting and quantification of levels of PKBα expression.**
4   Cell extracts were resolved by 7.5% SDS-PAGE and transferred to
5   Immobilon membranes (Millipore). Filters were blocked for 30 min
6   in a blocking buffer containing 5% skimmed milk in 1x TBS, 1%
7   Triton X-100 and 0.5% Tween 20, followed by a 2h incubation with
8   the 12CA5 supernatant 1000-fold diluted in the same buffer. The
9   secondary antibody was alkaline conjugated anti-mouse Ig from goat
10   (Southern Biotechnology Associates, Inc), 1000-fold diluted in the
11   blocking buffer. Detection was performed using AP colour
12   development reagents from Bio-Rad according to the manufacturer's
13   instructions. Quantification of levels of PKBα expression was
14   achieved by chemiluminescence, using fluroisothiocyanante-labelled
15   antimouse IgG from goat as the secondary antibody and the Storm
16   840/860 and ImageQuant software from Molecular Dynamics.

17

18   All peptides used to assay PKBα, and TTYADFIASGRTGRRNAIHD (the
19   specific peptide inhibitor of cyclic AMP dependent protein kinase
20   - PKI) were synthesised on an Applied Biosystems 431A peptide
21   synthesizer. Their purity (> 95%) was established by HPLC and
22   electrospray mass spectrometry, and their concentrations were
23   determined by quantitative amino acid analysis.

24

25   **Preparation and assay of PKBα.** The construction of cytomegalovirus
26   vectors (pCMV5) of the human haemagglutonin epitope-tagged wild
27   type _(HA-PKBα) was described previously (Alessi et al., 1996).
28   293 cells grown on 10 cm dishes were transfected with a DNA
29   construct expressing HA-PKBα using a modified calcium phosphate
30   procedure (Alessi et al., 1996). The cells were deprived of serum
31   for 16h prior to lysis and, where indicated, were stimulated for
32   10 min in the presence of 50 ng/ml IGF-1 to activate PKBα. The
33   cells were lysed in 1.0 ml ice-cold Buffer A (50 mM Tris/HCl pH
34   7.5, 1 mM EDTA 1 mM EGTA, 1% (by vol) Triton X-100, 1 mM sodium
35   orthovanadate, 10 mM sodium β-glycerophosphate, 50 mM NaF, 5 mM
36   sodium pyrophosphate, 1 µM Microcystin-LR, 0.27 M sucrose, 1 mM
37   benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 10 µg/ml
38   leupeptin, and 0.1 % (by vol) 2-mercaptoethanol) the lysate
39   centrifuged at 4°C for 10 min at 13, 000 x g. and the supernatant
40   obtained from one 10 cm dish of cells (2-3 mg protein) was
41   incubated for 60 min on a shaking platform with 20 µl of protein
42   G-Sepharose coupled to 10 µg of HA monoclonal antibody. The
43   suspension was centrifuged for 1 min at 13, 000 x g, the Protein

1   G-Sepharose-antibody-HA-PKBα complex washed twice with 1.0 ml of
2   Buffer A containing 0.5 M NaCl, and twice with Buffer B (50 mM
3   Tris/HCl pH 7.5, 0.1 mM EGTA, 0.01% (by vol) Brij-35 and 0.1% (by
4   vol) 2-mercaptoethanol). The PKBα immunoprecipitates were diluted
5   in Buffer B to an activity of 2.0 U/ml towards the Crosstide
6   peptide GRPRTSSFAEG and 0.1 ml aliquots snap frozen in liquid
7   nitrogen and stored at -80 oC. No significant loss of PKBα
8   activity occurred upon thawing the PKBα immunoprecipitates or
9   during storage at -80oC for up to 3 months . The standard
10 PKBα_assay (50 μl) contained: 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA,
11 0.1% (by vol) 2-mercaptoethanol, 2.5 μM PKI, 0.2 U/ml PKBα,
12 Crosstide (30 μM), 10 mM magnesium acetate and 0.1 mM [γ^{32}P]ATP
13 (100-200 cpm/pmol). The assays were carried out for 15 min at
14 30oC, the assay tubes being agitated continuously to keep the
15 immunoprecipitate in suspension, then terminated and analysed as
16 described (Alessi et al., 1995). One unit of activity was that
17 amount of enzyme which catalysed the phosphorylation of 1 nmol of
18 Crosstide in 1 min. The phosphorylation of other peptides, histone
19 H2B and MBP were carried out in an identical manner. All the
20 Crosstide activity in HA-PKBα immunoprecipitates is catalysed by
21 PKBα (see Results) and the PKBα concentration in the
22 immunoprecipitates was estimated by densitometric scanning of
23 Coomassie blue-stained polyacrylamide gels, using bovine serum
24 albumin as a standard.   Protein concentrations were determined by
25 the method of Bradford using bovine serum albumin as standard
26 (Bradford et al., 1976). Michaelis constants (Km) and Vmax values
27 were determined from double reciprocal plots of 1/V against 1/S,
28 where V is the initial rate of phosphorylation, and S is the
29 substrate concentration. The standard errors for all reported
30 kinetic constants were within <$\pm$ 20%, and the data is reported as
31 mean values for 3 independent determinations. Fig 16 shows the
32 results relative to those obtained for unstimulated PKBα.
33
34 **Tryptic digestion of histone 2B phosphorylated by PKBα.** Histone
35 H2B (30 μM) was phosphorylated with 0.2 U/ml HA-PKBα. After 60 min
36 0.2 vol of 100% (by mass) trichloroacetic acid was added, and the
37 sample incubated for 1 h on ice. The suspension was centrifuged
38 for 10 min at 13, 000 x g, the supernatant discarded and the
39 pellet washed five times with 0.2 ml of ice cold acetone. The
40 pellet was resuspended in 0.3 ml of 50 mM Tris/HCl pH 8.0, 0.1%
41 (by vol) reduced Triton-X100 containing 2 μg of alkylated trypsin
42 and, after incubation for 16 h at 30oC, the digest was centrifuged
43 for 5 min at 13, 000 x g. The supernatant, containing 95% of the

1 ^{32}P-radioactivity, was chromatographed on a Vydac C18 column

2 equilibrated with 0.1% (by vol) trifluoroacetic acid (TFA) in

3 water. With reference to the results shown in Fig 17, the columns

4 were developed with a linear acetonitrile gradient (diagonal line)

5 at a flow rate of 0.8 ml / min and fractions of 0.4 ml were

6 collected.   (A) Tryptic peptide map of ^{32}P-labelled histone H2B,

7 70% of the radioactivity applied to the column was recovered from

8 the major ^{32}P-peptide eluting at 19.5% acetonitrile. (B) A portion

9 of the major ^{32}P-peptide (50 pmol) was analysed on an Applied

10 Biosystems 476A sequencer, and the phenylthiohydantoin (Pth) amino

11 acids identified after each cycle of Edman degradation are shown

12 using the single letter code for amino acids. A portion of the

13 major ^{32}P-peptide (1000 cpm) was then coupled covalently to a

14 Sequelon arylamine membrane and analysed on an Applied Biosystems

15 470A sequencer using the modified programme described in (Stokoe

16 et al., 1992). ^{32}P radioactivity was measured after each cycle of

17 Edman degradation.

18

19

58

Table 7.1

Molecular basis for the substrate specificity of PKBα

The phosphorylated residue is shown in boldface type, the altered residue is underlined. V(100 µM) is the relative rate of phosphorylation at 0.1 mM peptide relative to peptide 1. ND, not determined. *An alanine residue was added to the C-terminal of the peptide RPRTSSP, since we have experienced difficulty in synthesing peptides terminating in proline.

A	Peptides	Km (µM)	Vmax (U/mg)	V(0.1 mM)
1.	GRPRTSSFAEG	4	250	100
2.	RPRTSSFA	8	305	109
3.	GRPRTSSF	8	385	129
4.	RPRTSSF	5	260	105
5.	RPRTSTF	30	243	78
6.	RPRTSAF	–	0	
7.	PRTSSF	–	0	
8.	RPRTSS	>500	ND	2
9.	KPRTSSF	>500	ND	4
10.	RPKTSSF	>500	ND	2
B				
1.	RPRTSSF	5	260	105
2.	RPRTSSL	8	278	104
3.	RPRTSSV	21	300	102
4.	RPRTSSA	250	265	30
5.	RPRTSSK	80	308	67
6.	RPRTSSE	>500	ND	9
7.	RPRTSSPA*	–	0	
C				
1.	RPRTSSF	5	260	105
2.	RPRASSF	12	230	89
3.	RPRVSSF	25	273	77
4.	RPRGSSF	60	163	37
5.	RPRNSSF	>500	ND	21
6.	RPRTASF	20	213	83
7.	RPRTGSF	25	233	77
8.	RPRTVSF	30	365	89
9.	RPRTNSF	30	300	81
10.	RPRAASF	25	215	77
11.	RPRGGSF	105	345	55
12.	RPRGASF	105	160	37
13.	RPRAGSF	49	114	70

Table 12 Comparison of the substrate specificities of PKBα, MAPKAP kinase-1, and p70S6 kinase. Peptides 1 and 2 are very good substrates for MAPKAP kinase-1 and p70 S6 kinase, and peptide 3 is a relatively specific substrate for p70 S6 kinase [16]. *Data reported previously [16]; ND, not determined.

A

Peptide	Protein kinase Bα		MAPKAP kinase-1		p70 S6 kinase	
	K_m (mM)	V_{max} (U/mg)	K_m (mM)	V_{max} (U/mg)	K_m (mM)	V_{max} (U/mg)
1. KKKNRTLSVA	185	270	0.2*	1550*	3.3*	890*
2. KKRNRTLSVA	80	300	0.7*	1800*	1.5*	1520*
3. KKRNKTLSVA	>500	ND	17*	840*	34*	760*
4. KKRNRTLTV	388	330	40*	270*	4.8*	1470*

B

Peptide	Protein kinase Bα		MAPKAP kinase-1		p70 S6 kinase	
	K_m (mM)	V_{max} (U/mg)	K_m (mM)	V_{max} (U/mg)	K_m (mM)	V_{max} (U/mg)
1. GRPRTSSFAEG	4	250	2	790	3	1270
2. RPRTSSF	5	260	12	840	125	705
3. RPRTSTF	30	240	>500	ND	211	590
4. RPRAASF	25	215	20	1020	>500	ND
5. RPRAATF	25	230	>500	ND	>500	ND

60

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32

33

1

2   Claims:

3   1   The use of a composition of PKB, its analogues,

4   isoforms, inhibitors, activators and/or the functional

5   equivalents thereof, to regulate glycogen metabolism

6   and/or protein synthesis.

7

8   2   The use of a composition of PKB, its analogues,

9   isoforms, inhibitors, activators and/or the functional

10   equivalents thereof, for the manufacture of a

11   medicament to regulate glycogen metabolism and/or

12   protein synthesis.

13

14   3   The use as claimed in claim 1 or claim 2, to

15   combat disease states where glycogen metabolism and/or

16   protein synthesis exhibits abnormality.

17

18   4   The use as claimed in claim 1, 2 or 3, to combat

19   diabetes.

20

21   5   The use as claimed in any preceding claim, to

22   combat cancer.

23

24   6   The use as claimed in claim 5, wherein the cancer

25   is breast, pancreatic or ovarian cancer.

26

27   7   The use as claimed in any preceding claim, wherein

28   the PKB is PKBα, β or γ, an analogue, isoform,

29   inhibitor, activator or a functional equivalent

30   thereof.

31

32   8   The use as claimed in any preceding claim, wherein

33   the PKB, its analogue, isoform, or functional

34   equivalent is modified at one or both of amino acids

35   308 and 473 by phosphorylation and/or mutation.

36

1   9   A composition of PKB, its analogues, isoforms,

2   inhibitors, activators and/or the functional

3   equivalents thereof.

4

5   10   A peptide having or including the amino acid

6   sequence Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd, where Xaa is

7   any amino acid, Yaa and Zaa are any amino acid, and Hyd

8   is a large hydrophobic residue, or a functional

9   equivalent of such a peptide.

10

11   11   A peptide as claimed in claim 10, wherein Hyd is

12   Phe or Leu, or a functional equivalent thereof.

13

14   12   A peptide as claimed in claim 10 or claim 11,

15   wherein Yaa or Zaa or both are an amino acid other than

16   glycine.

17

18   13   A peptide as claimed in claim 10, having the amino

19   acid sequence GRPRTSSFAEG, or a functional equivalent

20   thereof.

21

22   14   A method of identifying agents able to influence

23   the activity of GSK3, said method comprising:

24

25   a.   exposing a test substance to a substrate of GSK3;

26   and

27   b.   detecting whether said substrate has been

28   phosphorylated.

29

30   15   A method of identifying agents which influence the

31   activity of PKB, comprising:

32

33   a.   exposing a test substance to a sample containing

34   PKB, to form a mixture;

35   b.   exposing said mixture to a peptide as claimed in

36   claim 10, 11, 12 or 13; and

c.   detecting whether (and, optionally, to what
      extent) said peptide has been phosphorylated.

16   A method as claimed in claim 14 or 15, wherein the
extent of phosphorylation of the peptide is determined.

17   A method as claimed in claim 15, wherein the
phosphorylation state(s) of one or both of amino acids
308 and 473 on PKB is determined.

18   A method as claimed in any one of claims 14 to 17,
wherein the test substance is an analogue, isoform,
inhibitor, or activator of PKB.

19   A method as claimed in any one of claims 14 to 18,
wherein steps a or b (or both) are carried out in the
presence of divalent cations and ATP.

20   A method of treatment of the human or non-human
animal body, said method comprising administering PKB,
its analogues, inhibitors, stimulators or functional
equivalents thereof to said body.

21   A method as claimed in claim 20, to combat disease
states where glycogen metabolism and/or protein
synthesis exhibits abnormality.

22   A method as claimed in claim 20 or 21, to combat
diabetes.

23   A method as claimed in claim 20 or 22, to combat
cancer.

24   A method as claimed in claim 23, wherein the
cancer is breast, pancreatic or ovarian cancer.

25   A method as claimed in any one of claims 20 to 24,
wherein the PKB is PKBα, β or γ, an analogue, isoform,
inhibitor, activator or a functional equivalent
thereof.

26   An agent capable of influencing the activity of
PKB, its isoforms, analogues and/or functional
equivalents, by modifying amino acids 308 and/or 473 by
phosphorylation or mutation.

27   A method of determining the ability of a substance
to affect the activity or activation of PKB, the method
comprising exposing the substance to PKB and
phosphatidyl inositol polyphosphate and determining the
interaction between PKB and the phosphatidyl inositol
polyphosphate.

28   A method of determining the ability of a substance
to combat diabetes, cancer, or any disorder which
involves irregularity of protein synthesis or glycogen
metabolism, the method comprising exposing the
substance to PKB and phosphatidyl inositol
polyphosphate and determining the interaction between
PKB and the phosphatidyl inositol polyphosphate.

29   A method as claimed in claim 27 or claim 28,
wherein the interaction between PKB and the
phosphatidyl inositol polyphosphate is measured by
assessing the phosphorylation state of PKB.

30   A method as claimed in claim 29, wherein the
phosphorylation state of PKB at T308 and/or S473   is
assessed.

31   A method of identifying activators or inhibitors
of GSK3 comprising exposing the substance to be tested

1   to GSK3 and determining the state of activation of

2   GSK3.

3

4   32   A method as claimed in claim 31 wherein the state

5   of activation of GSK3 is determined by assessing its

6   phosphorylation.

7

8   33   A method of determining the suitability of a test

9   substance for use in combatting diabetes, cancer, or

10   any disorder which involves irregularity of protein

11   synthesis or glycogen metabolism, the method comprising

12   exposing the substance to be tested to GSK3 and

13   determining the state of activation of GSK3.

14

15   34   A method for screening for inhibitors or

16   activators of enzymes that catalyse the phosphorylation

17   of PKB, the method comprising exposing the substance to

18   be tested to

19   - one or more enzymes upstream of PKB;

20   - PKB; and (optionally)

21   - nucleoside triphosphate

22   and determining whether (and optionally to what extent)

23   the PKB has been phosphorylated on T308 and/or S473.

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Fig. 1a

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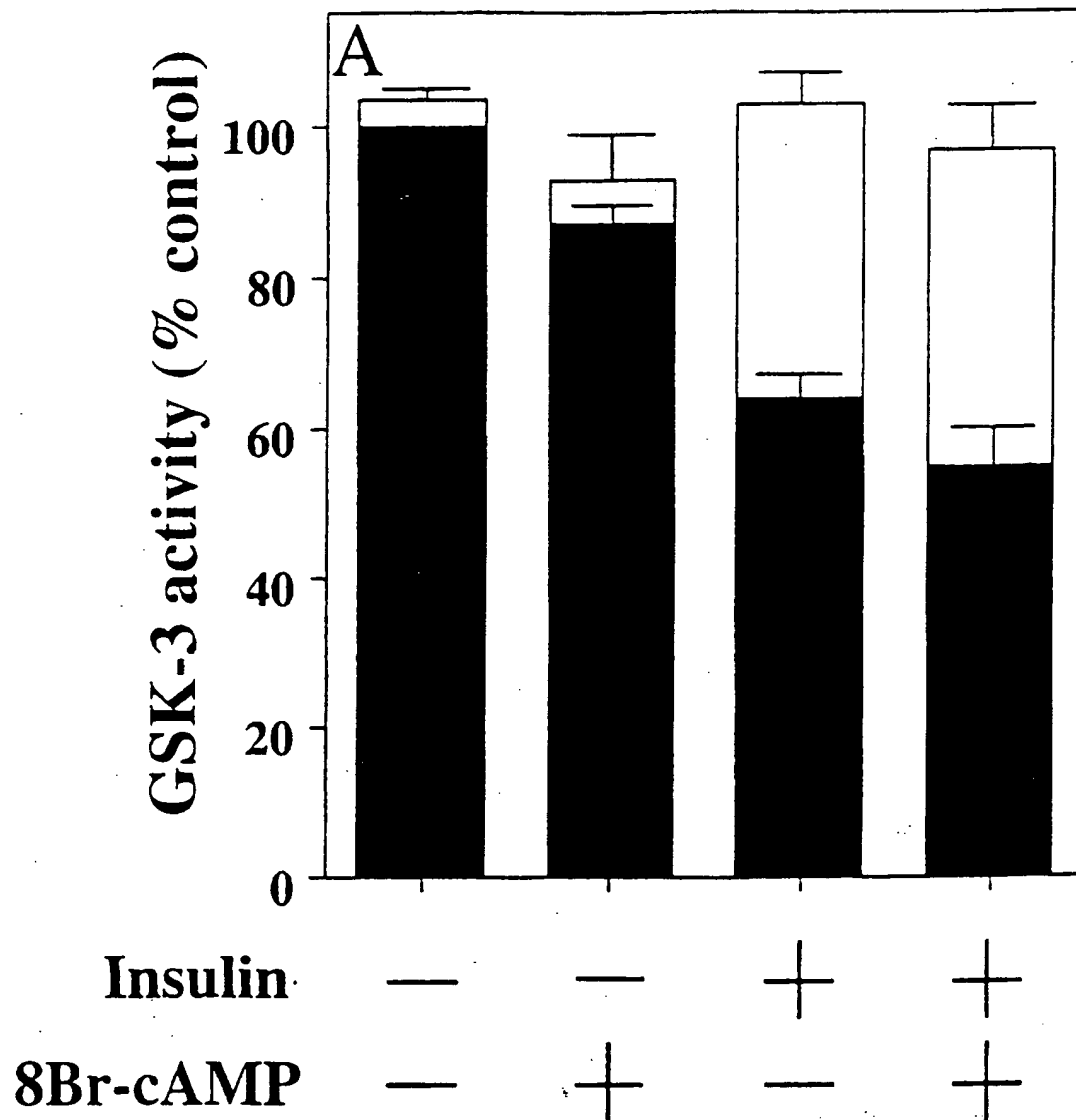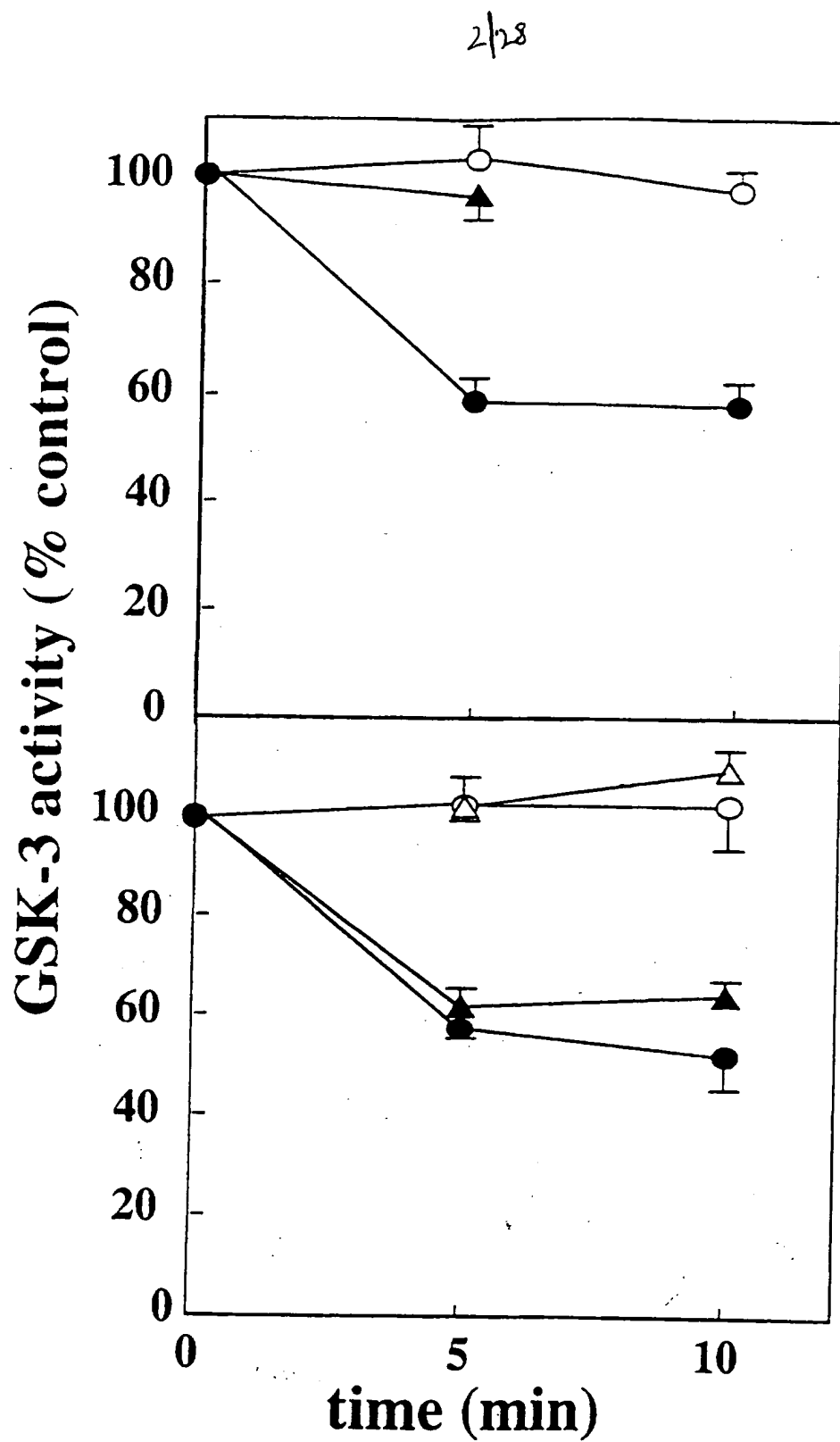
Figs. 1b & 1c
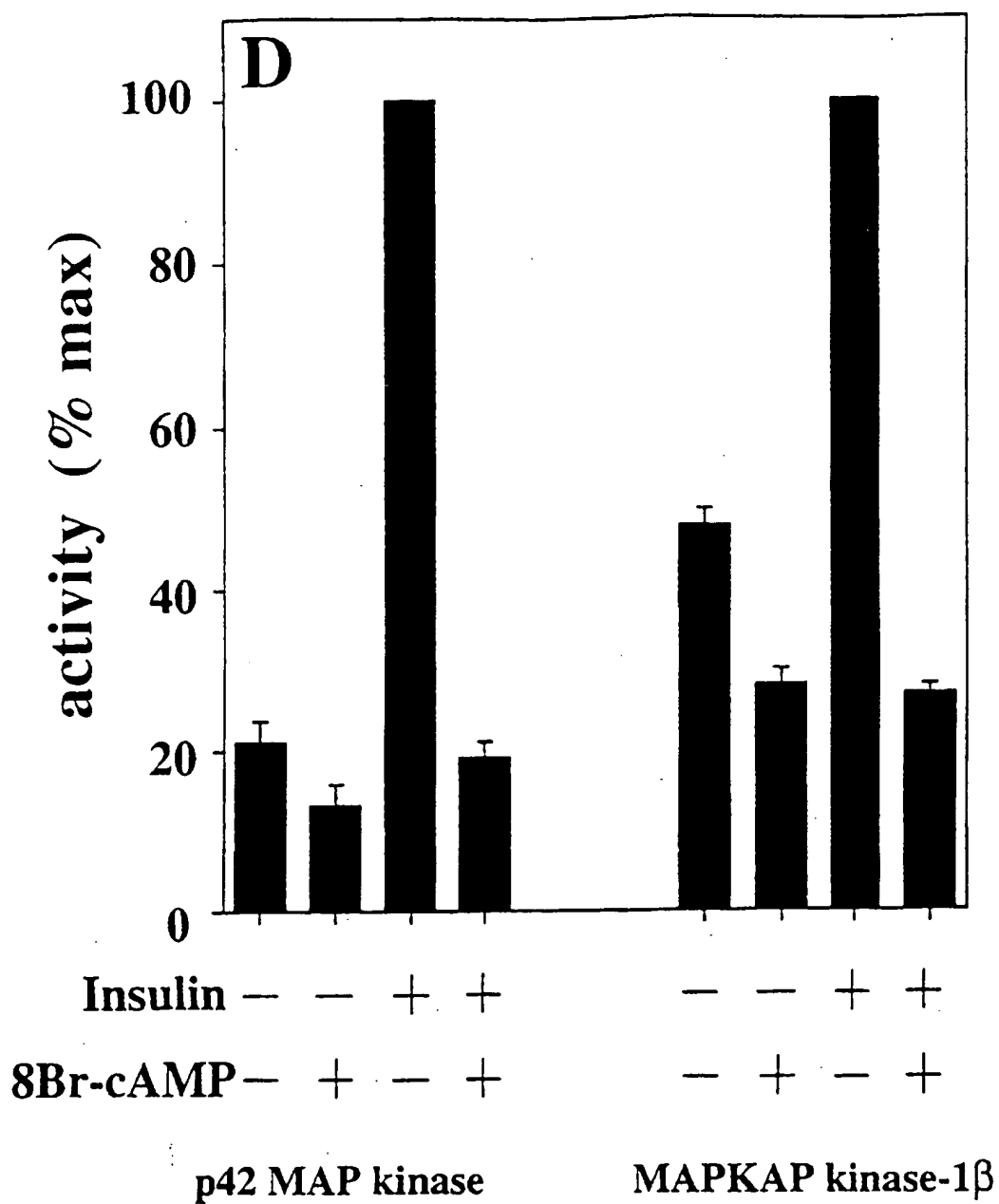
Fig. 1d

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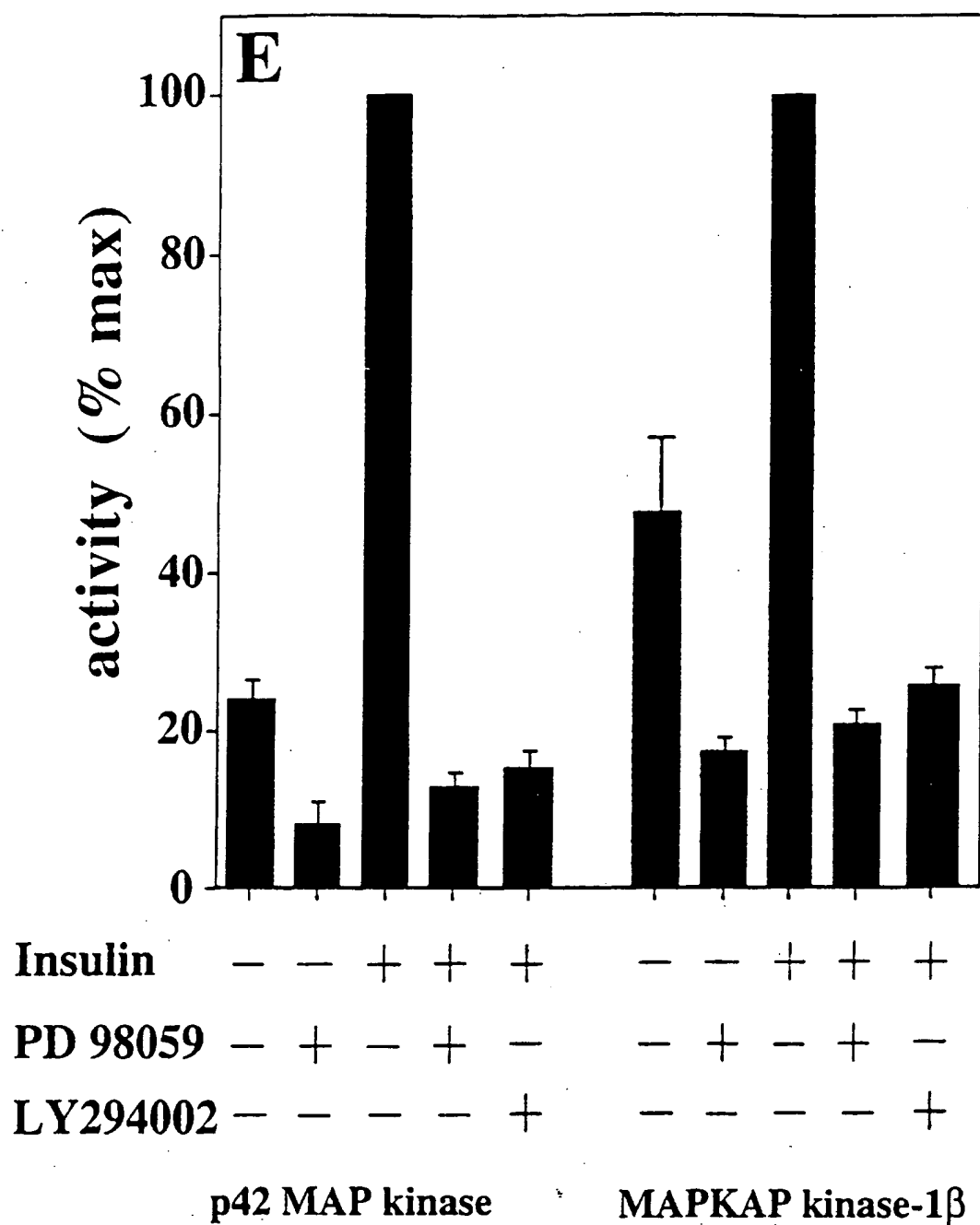
Fig. 1e

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Fig. 2a

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Figs. 2b, 2c & 2d

Fig. 3a
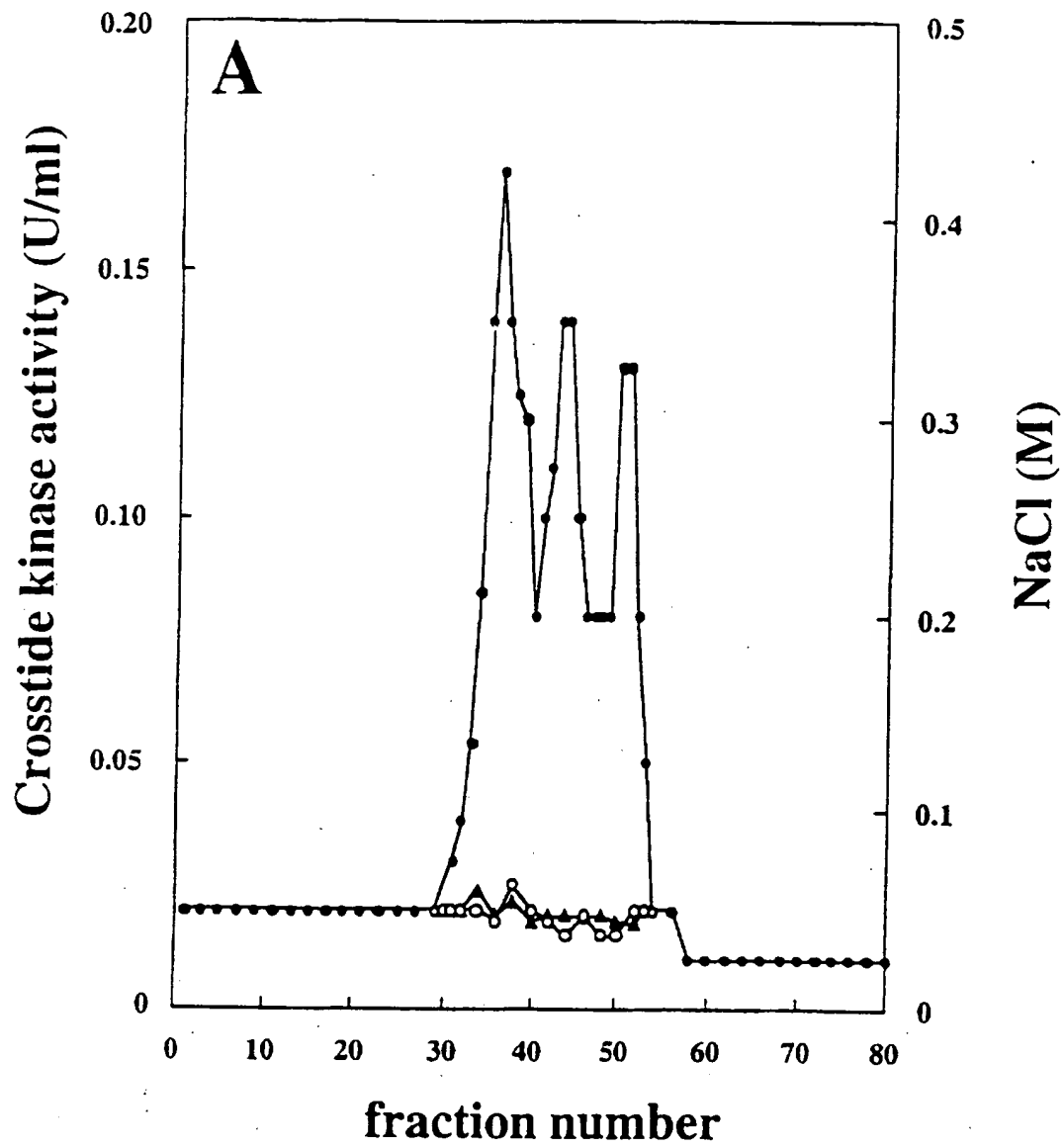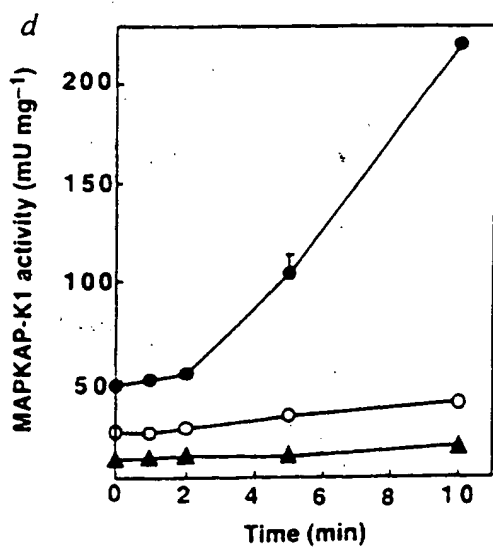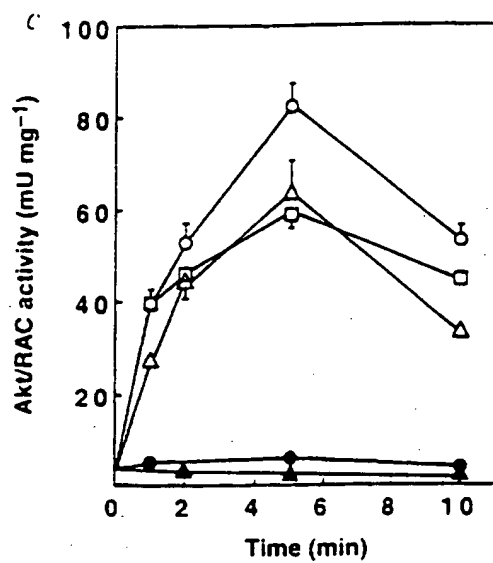
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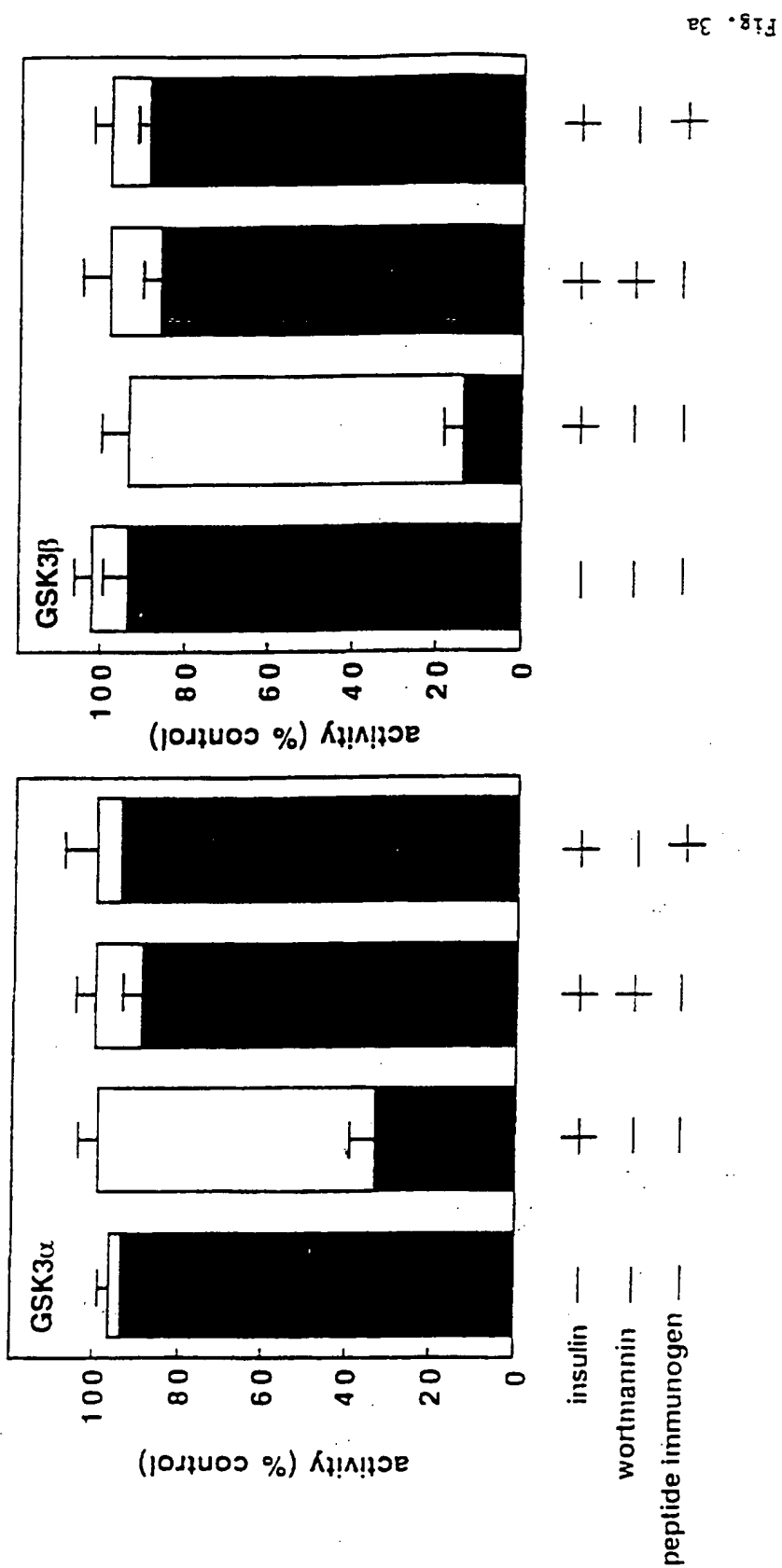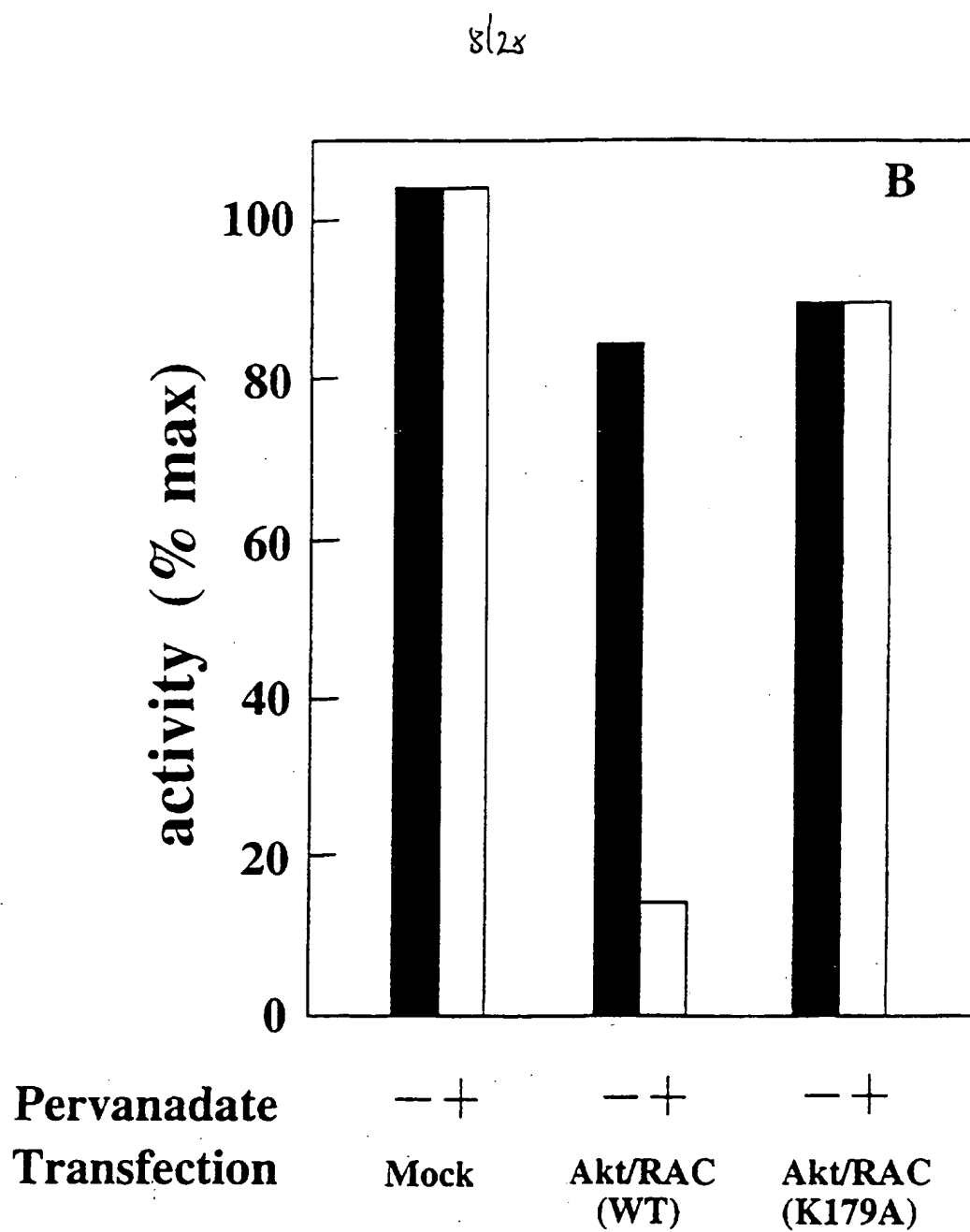
Fig. 3b

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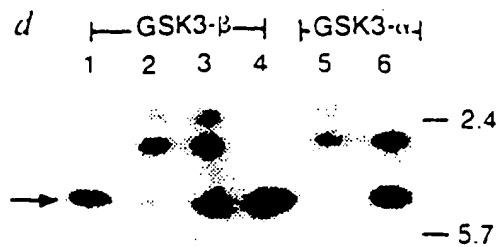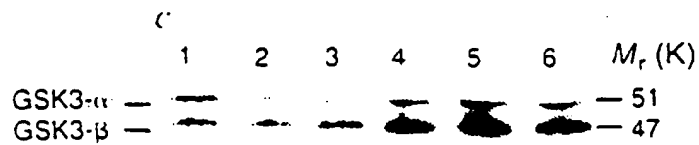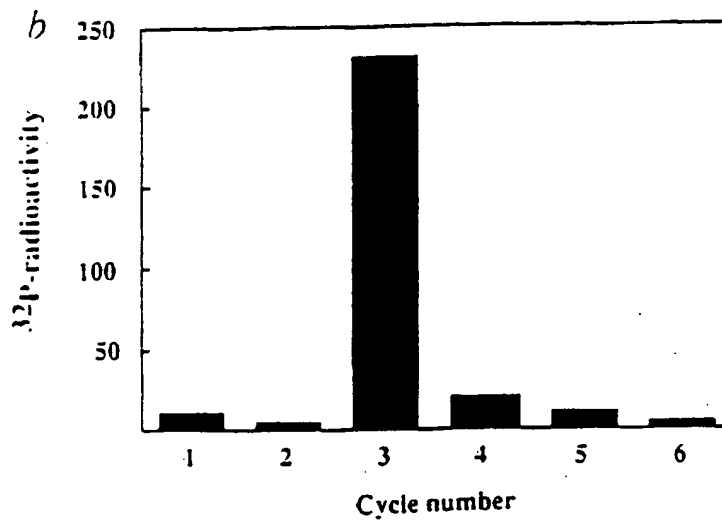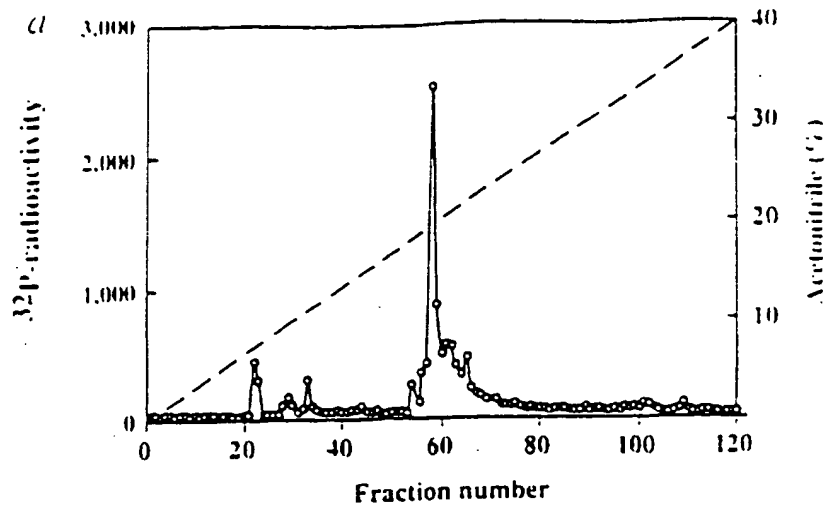
Figs. 4a, 4b, 4c & 4d

Fig. 5
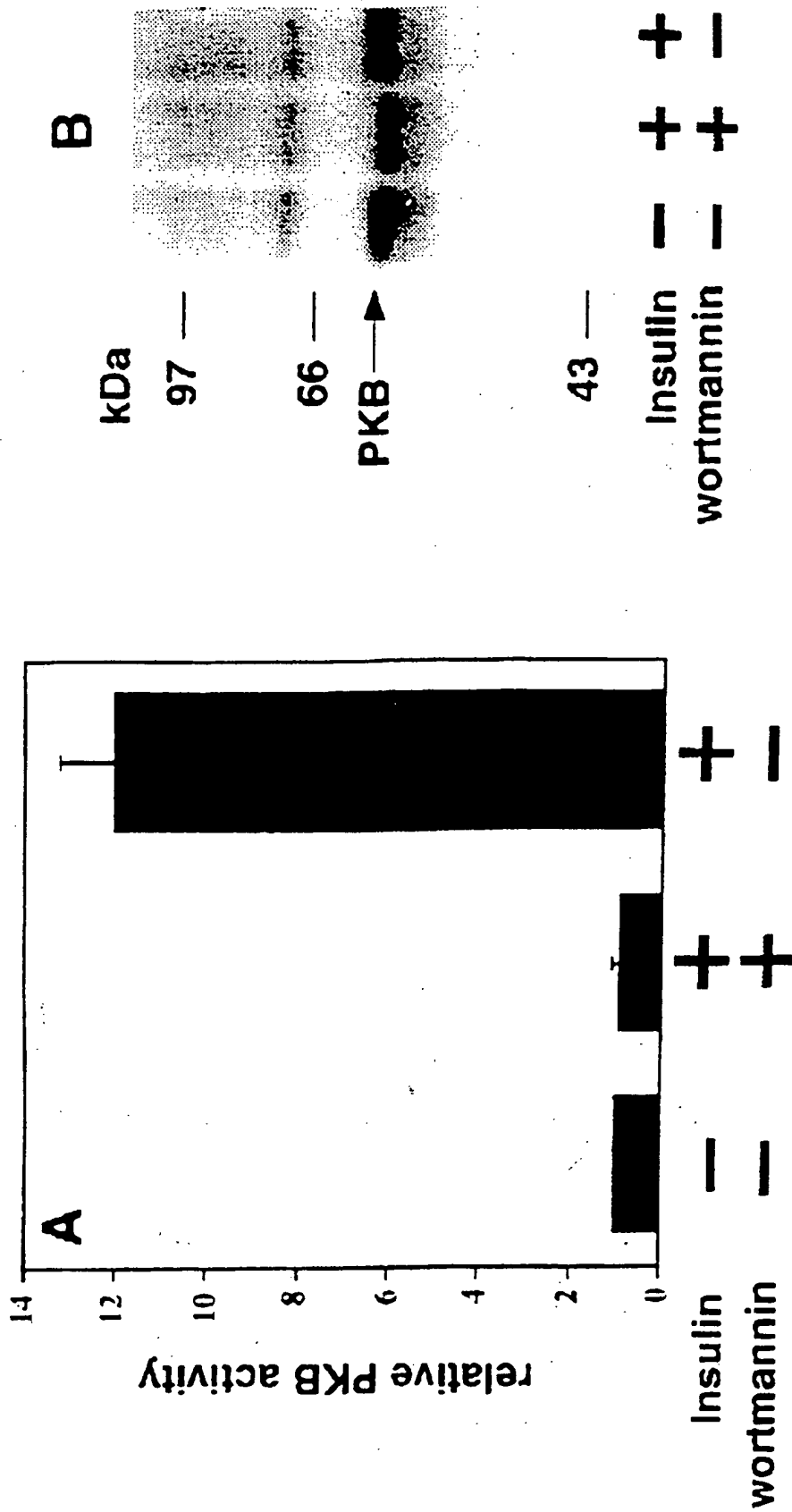
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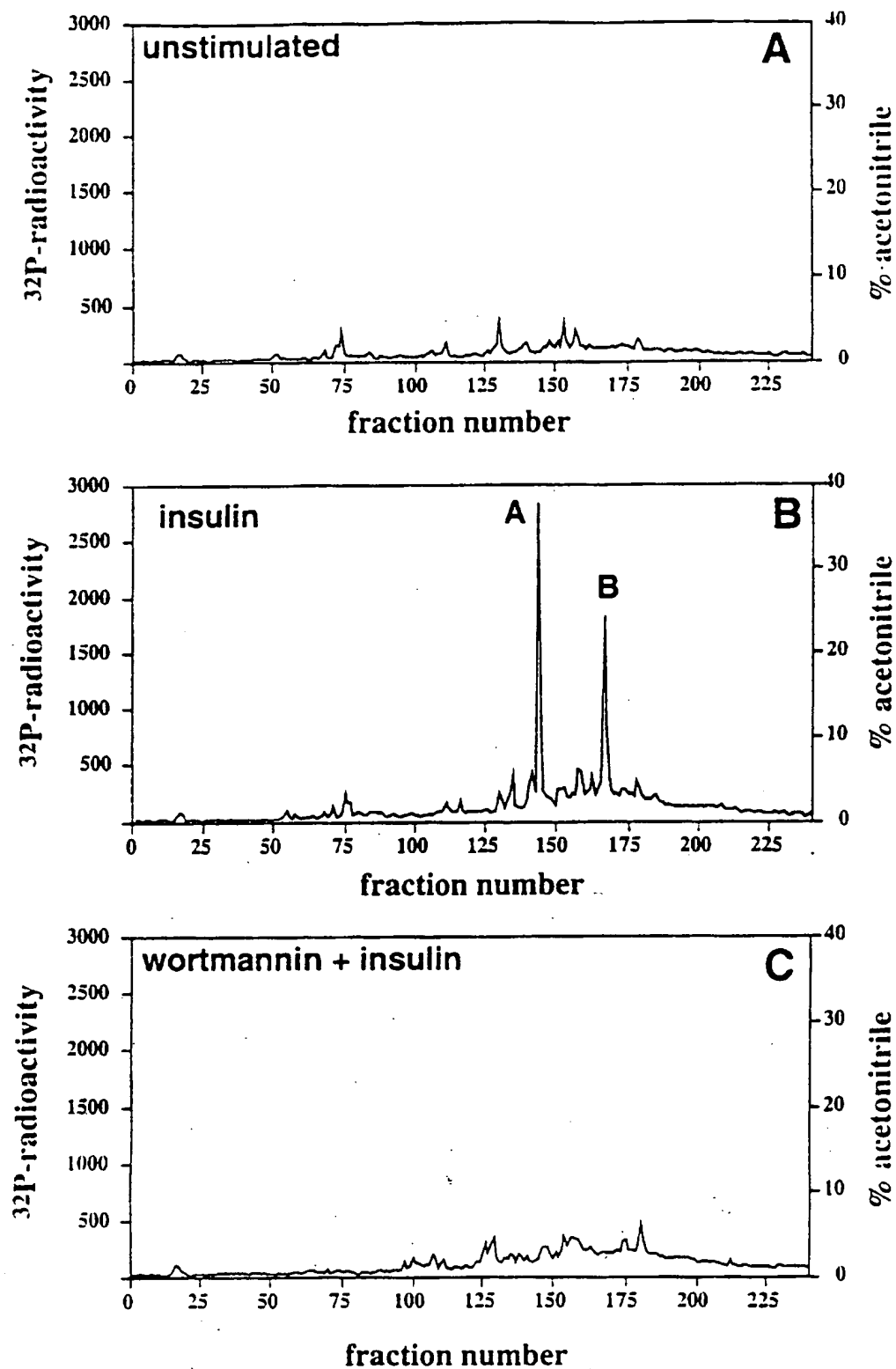
Fig. 6
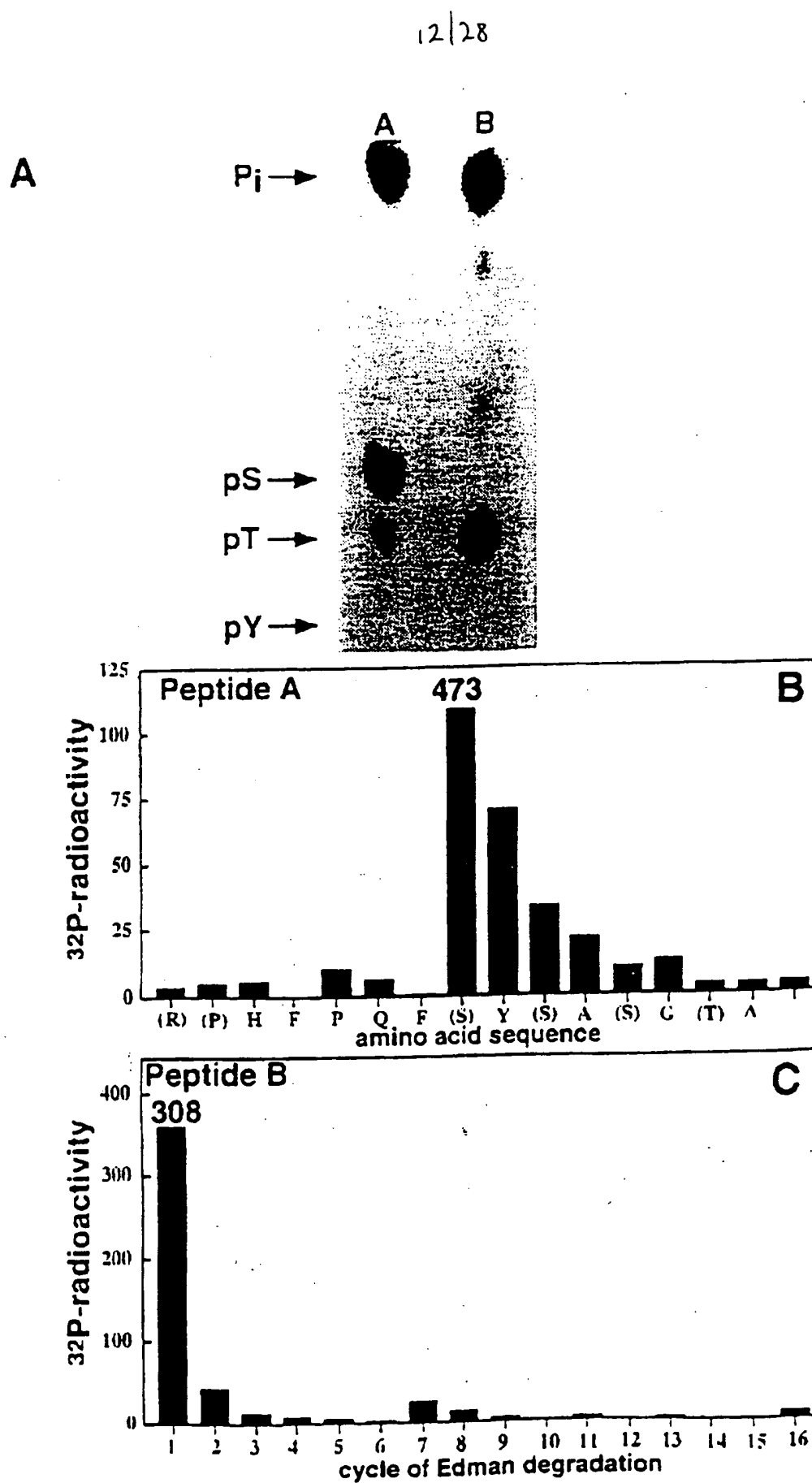
Fig. 7

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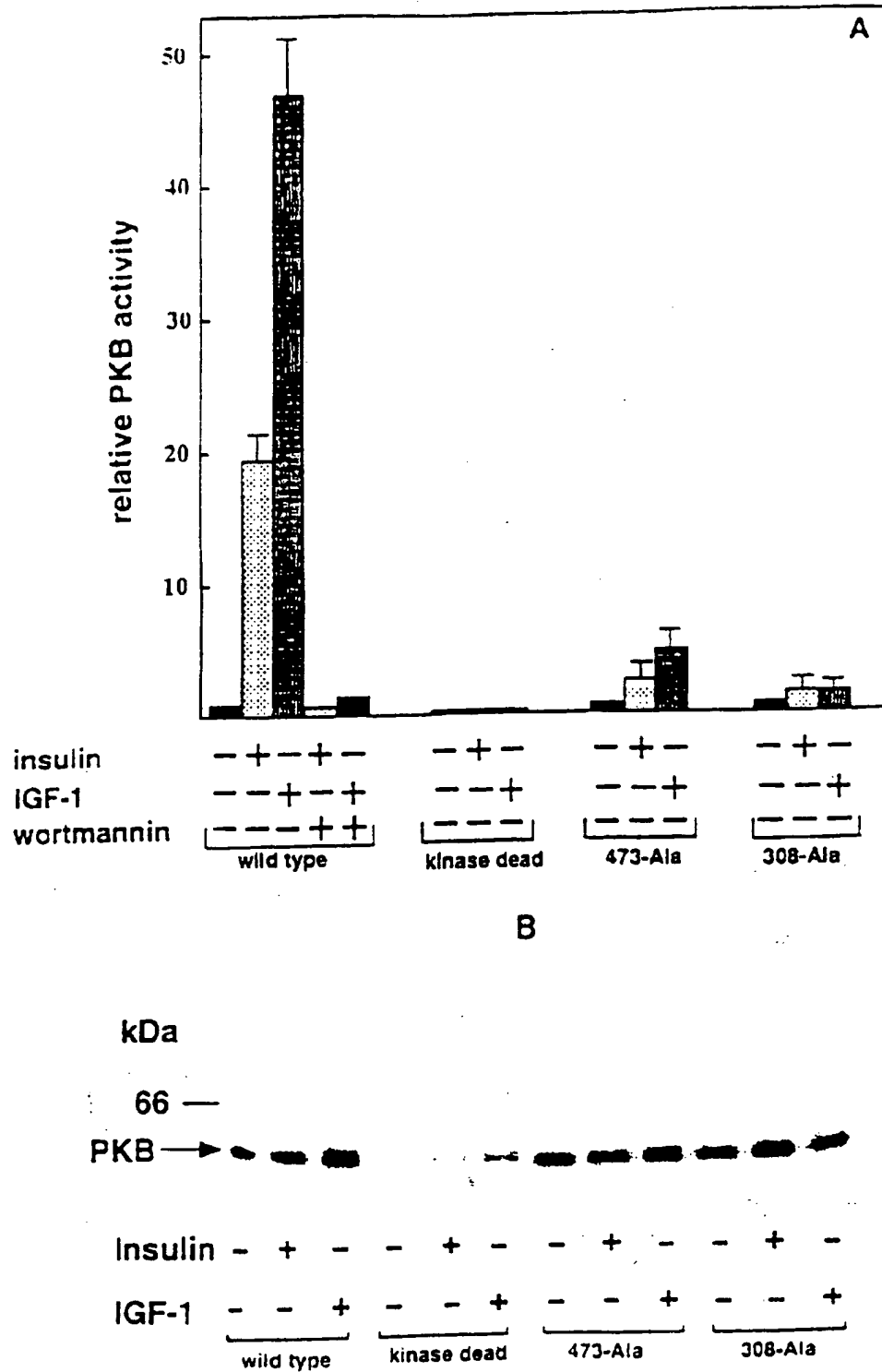
Fig. 8

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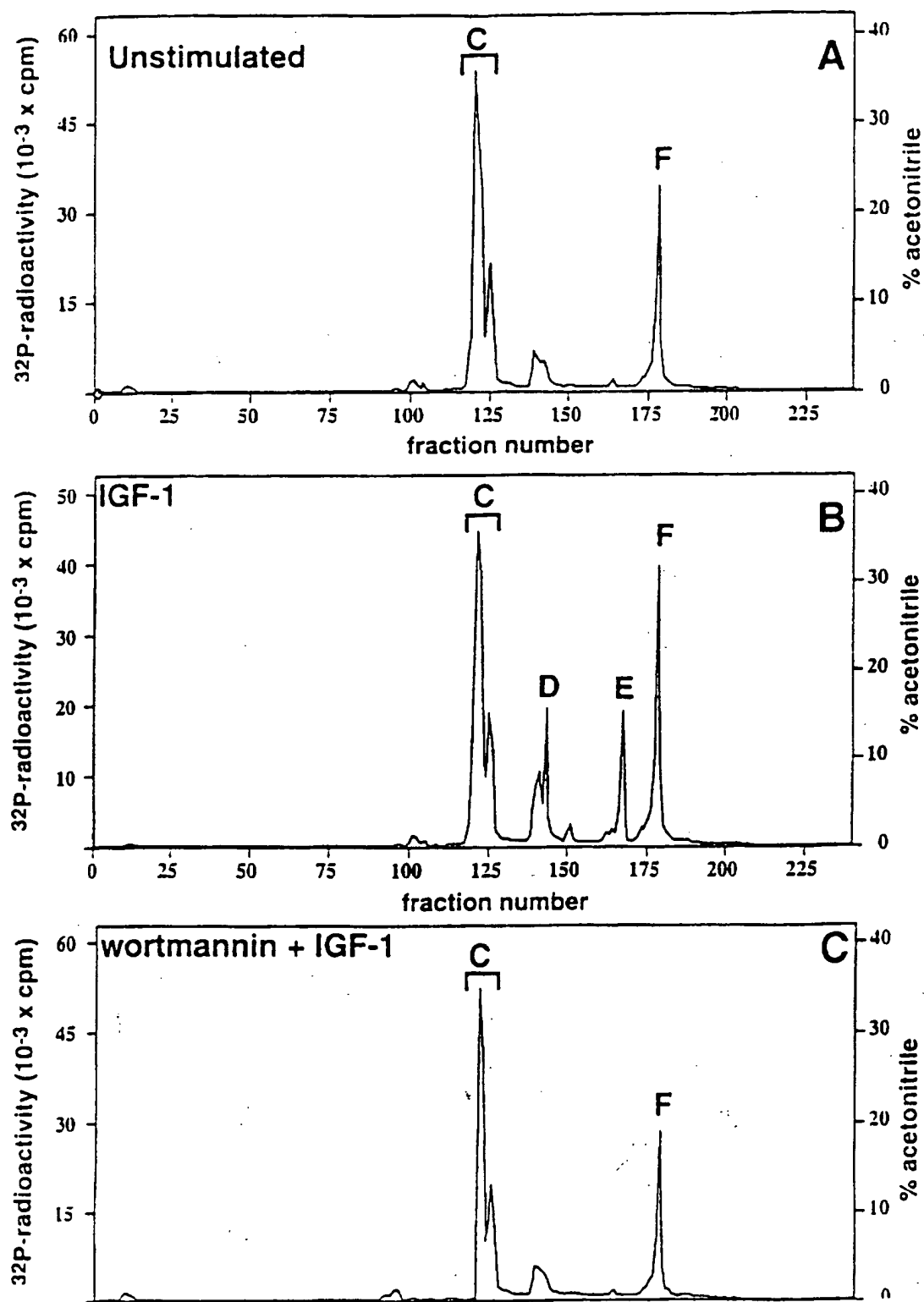
Fig. 9

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Fig. 10
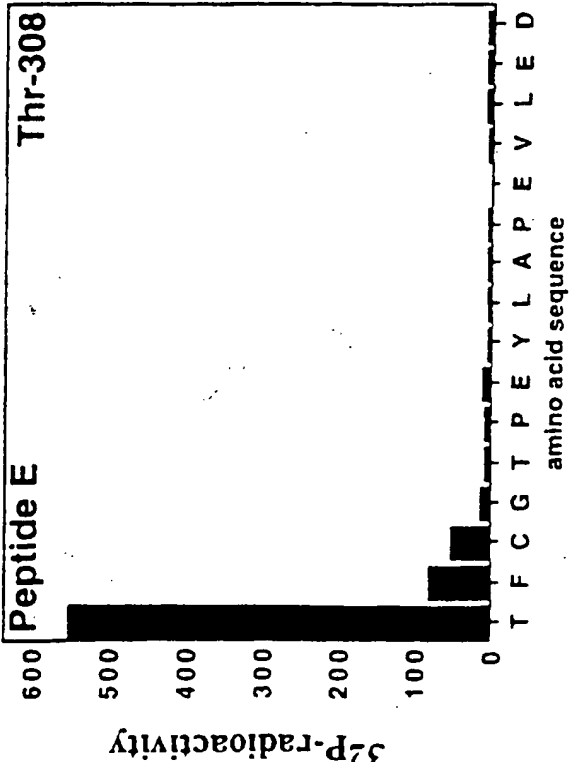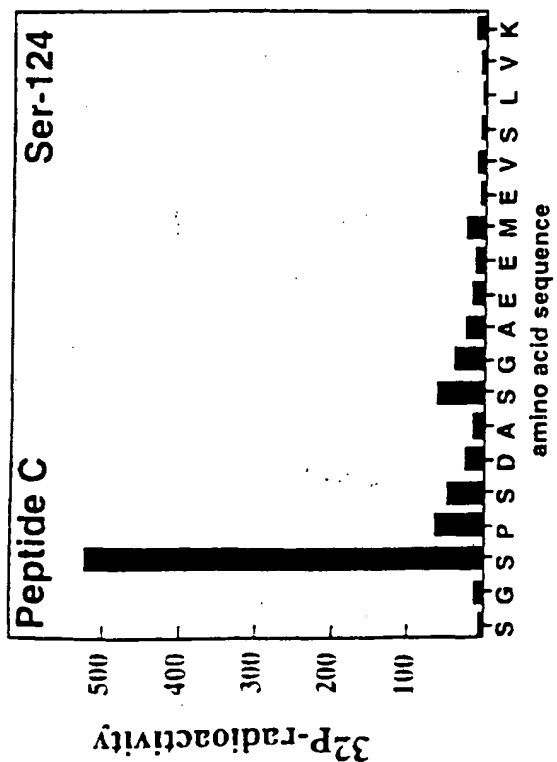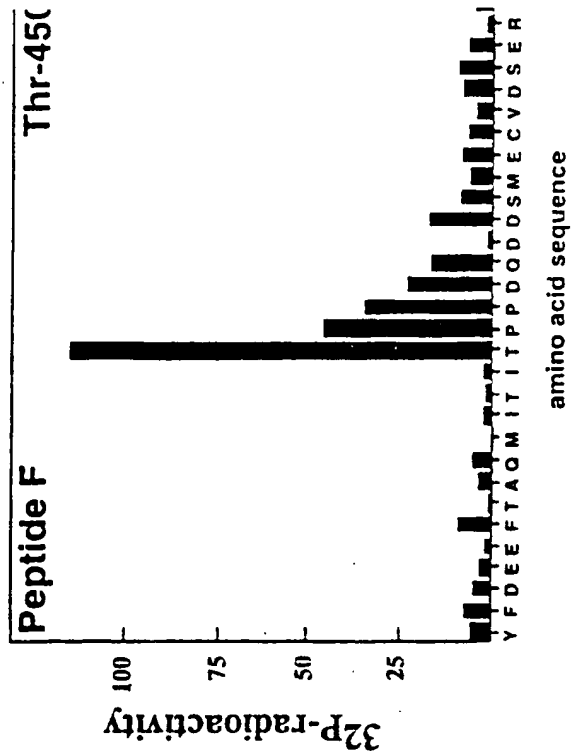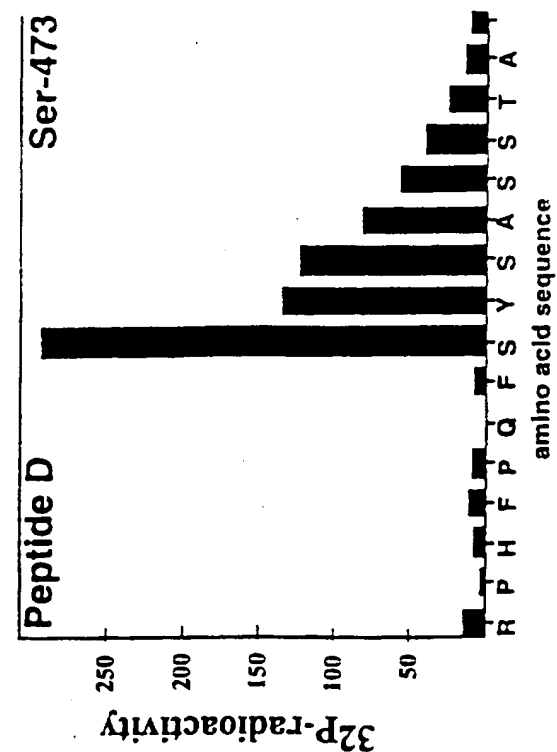
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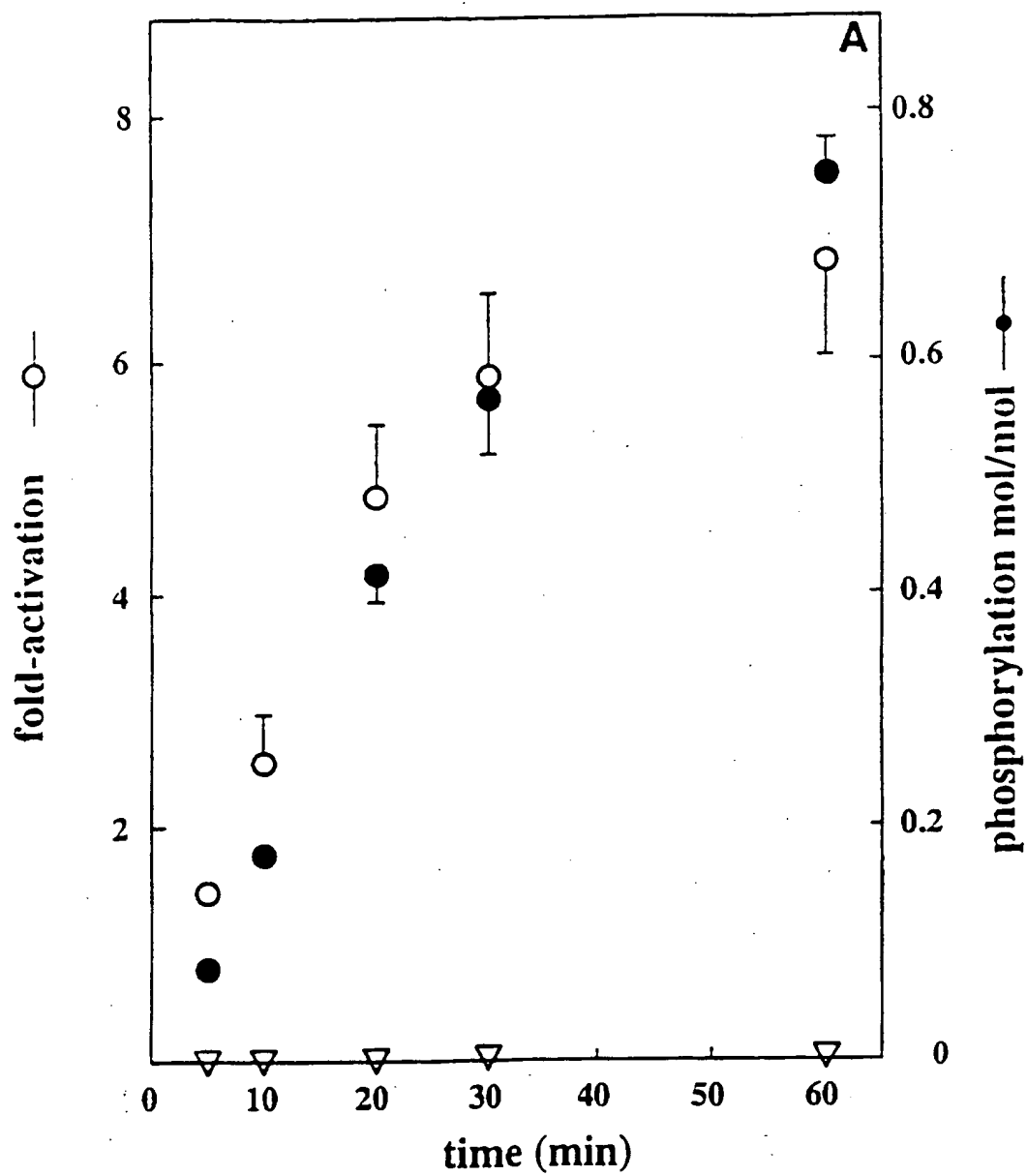
Fig. 11a

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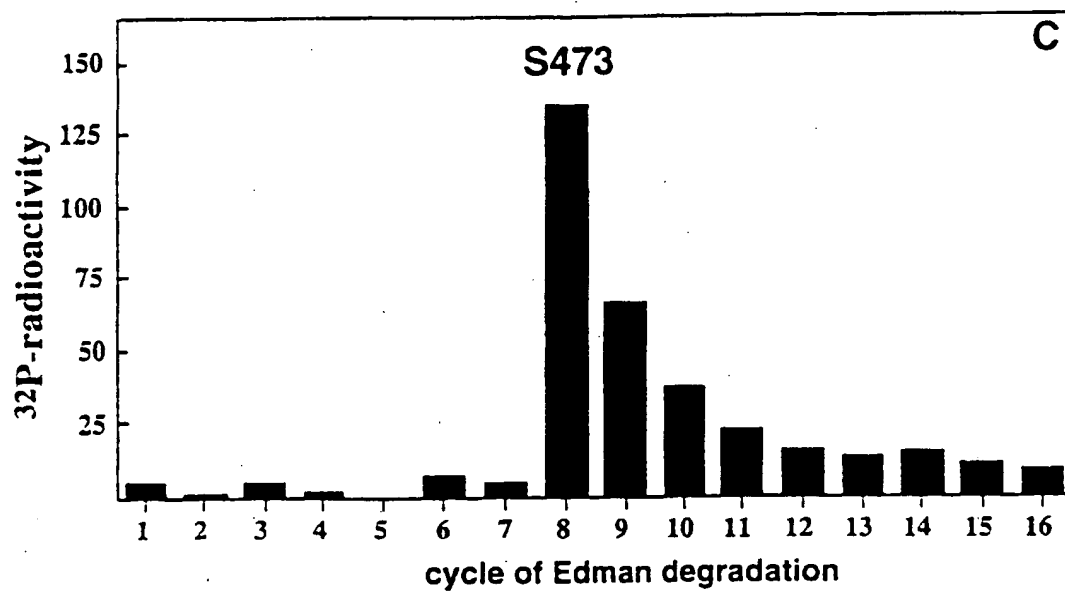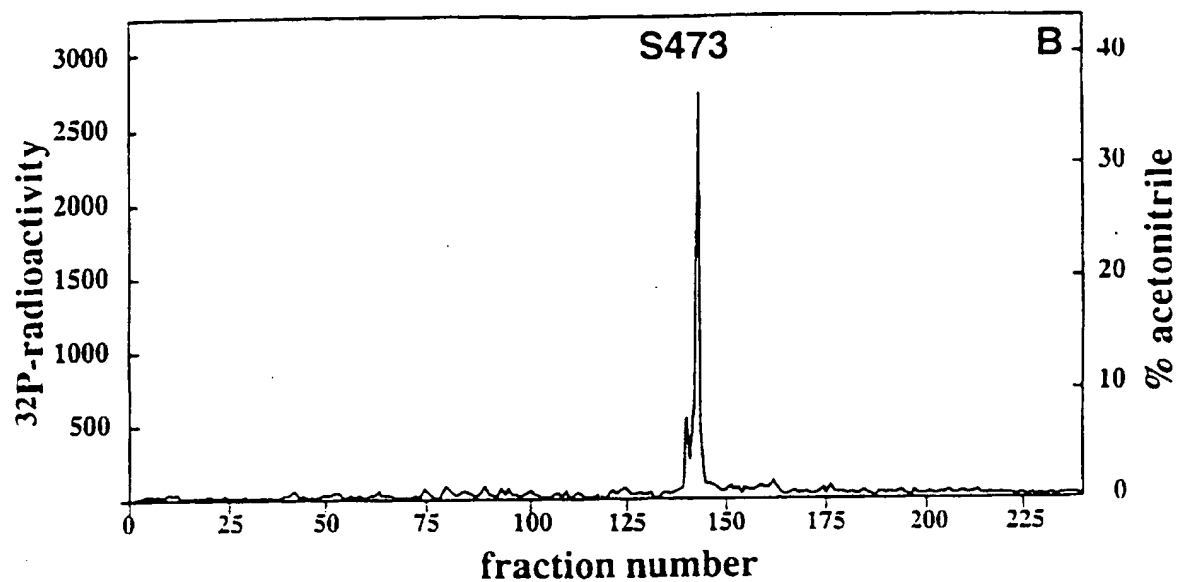
Figs. 11b & 11c

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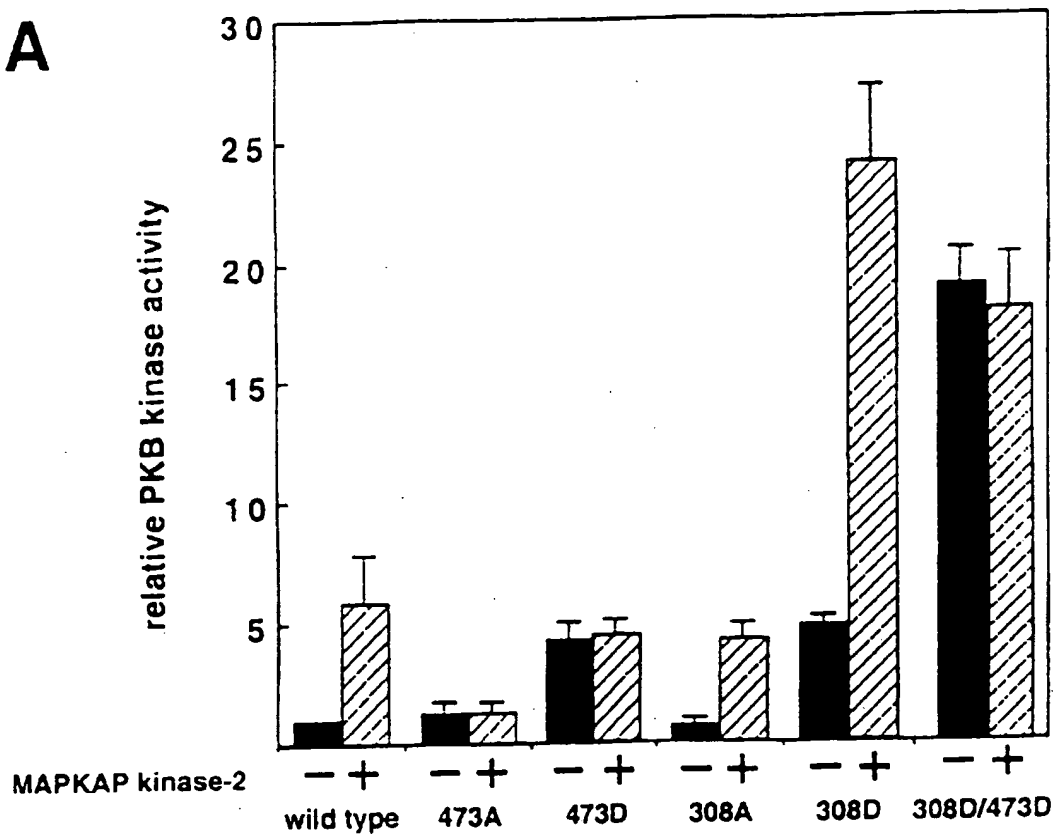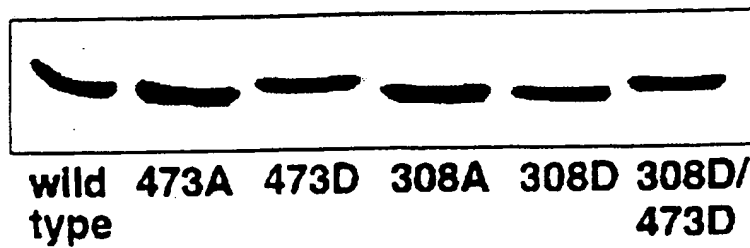
Figs. 12a & 12b

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A

B

Figs. 13a & 13b

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Fig. 14

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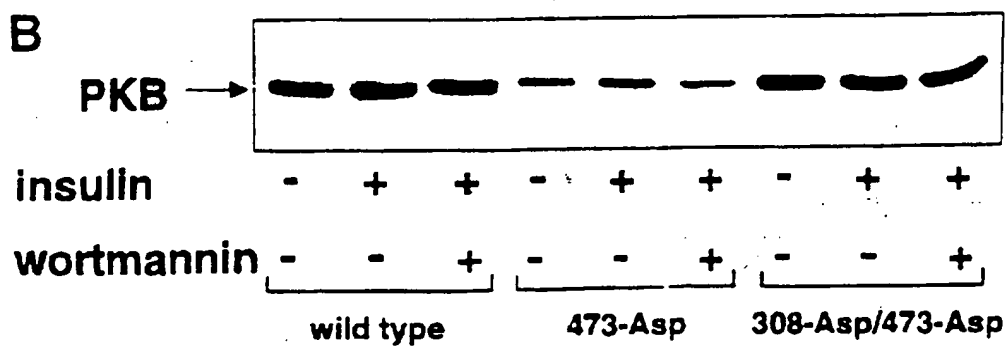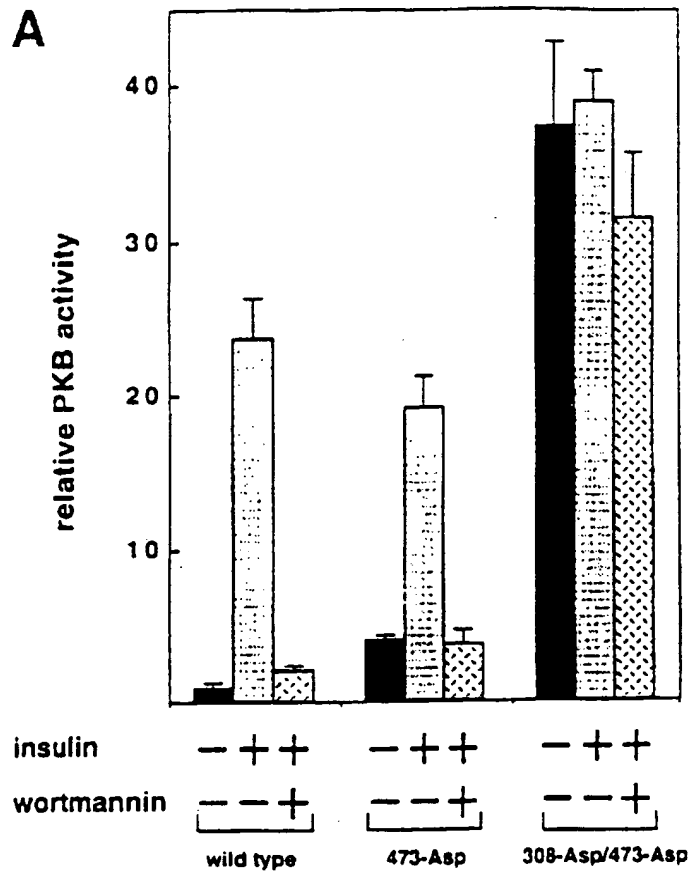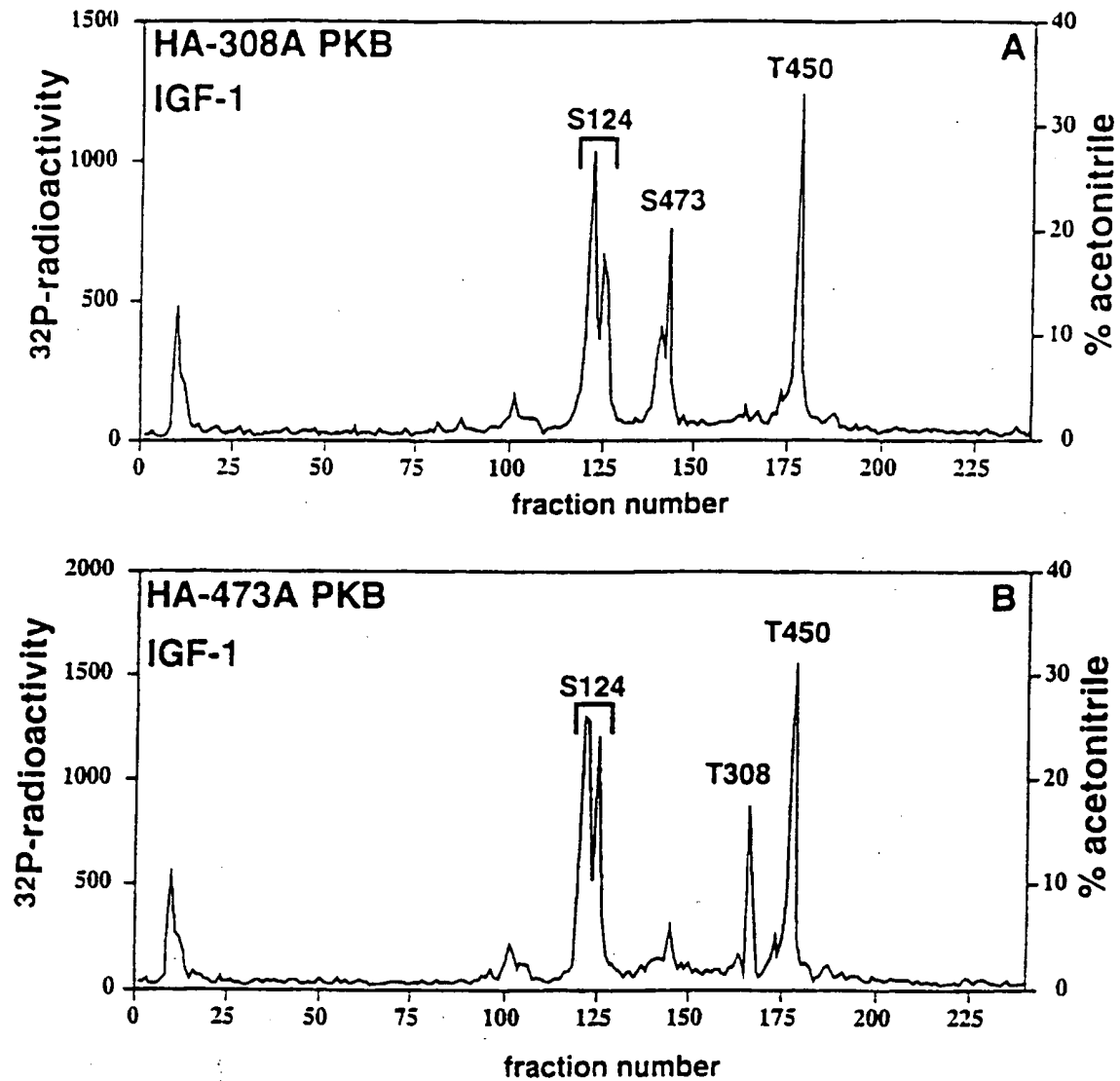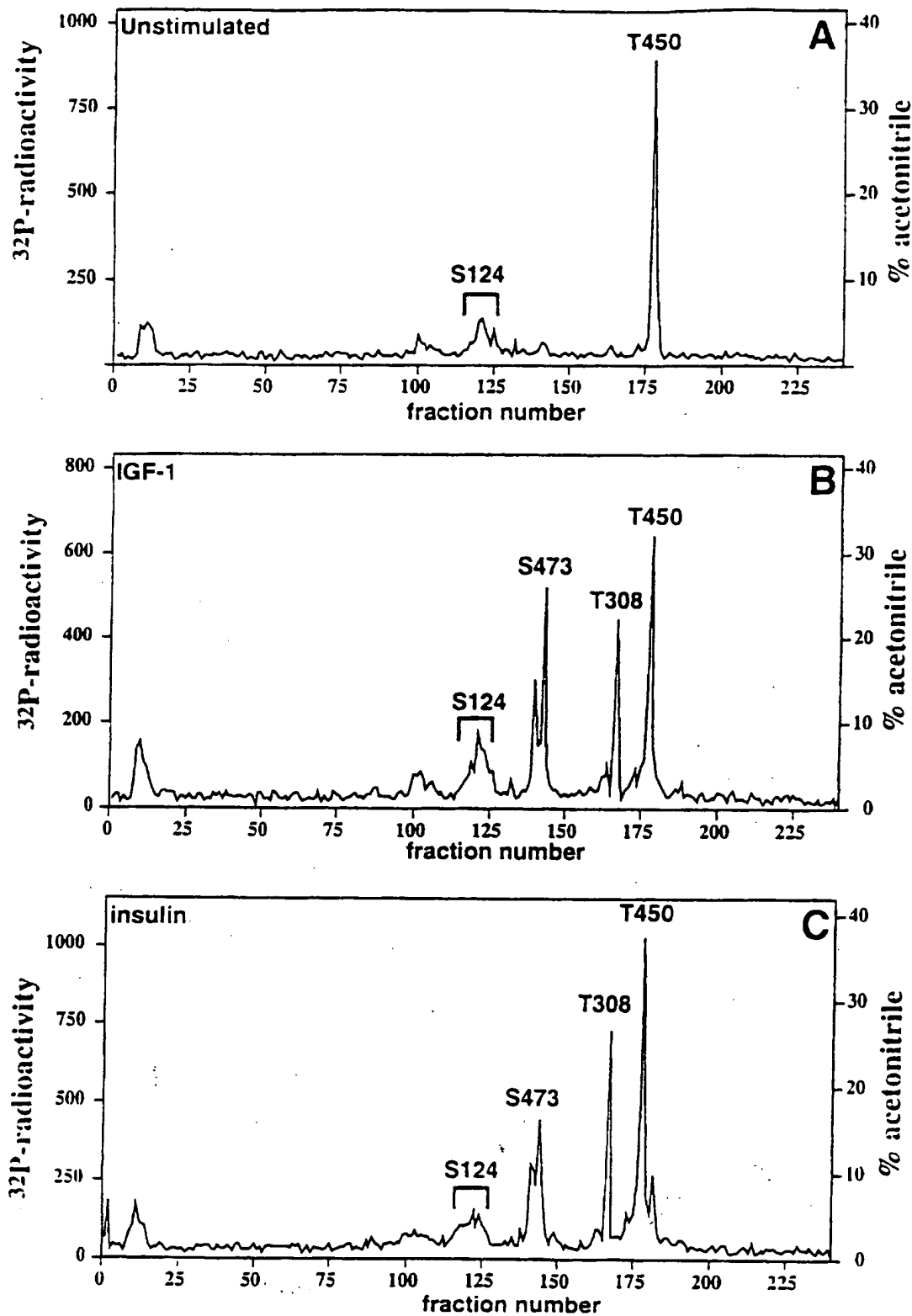
Fig. 15
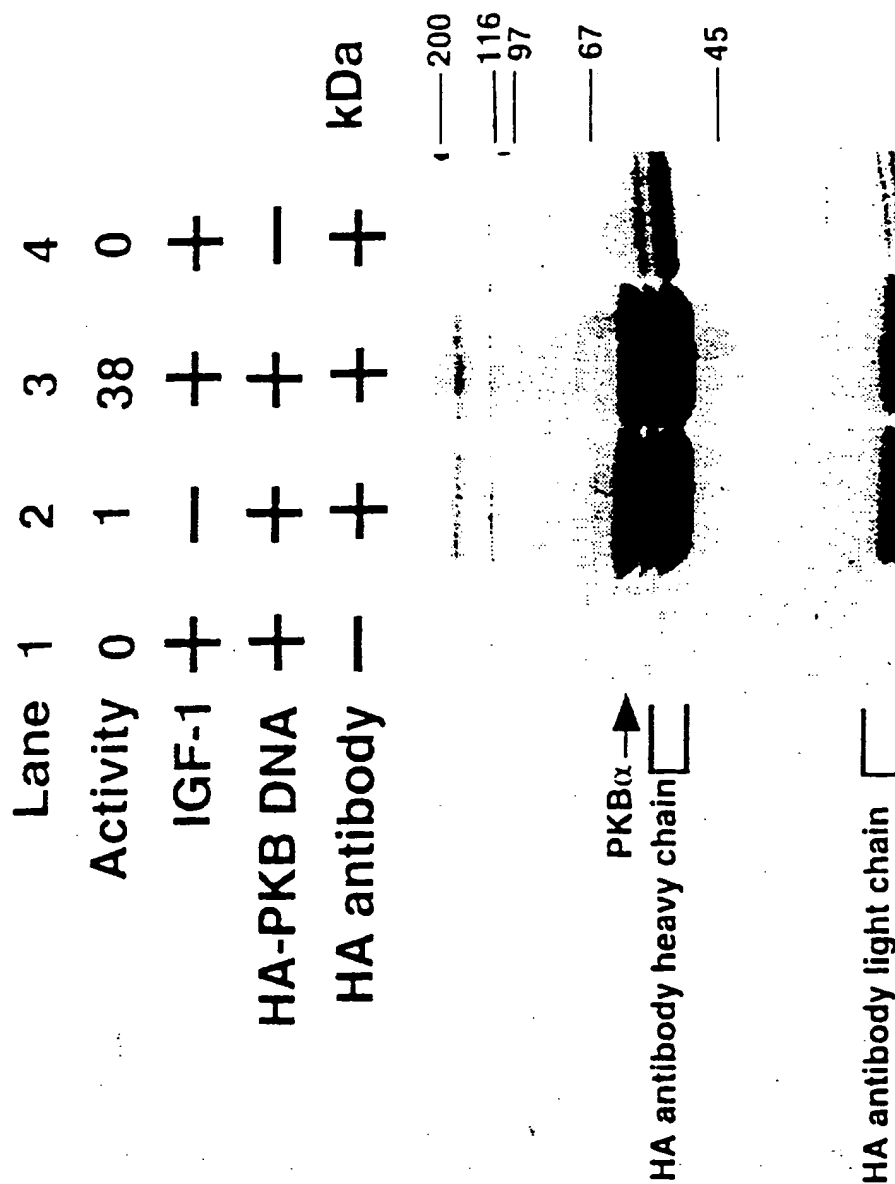
Fig. 16

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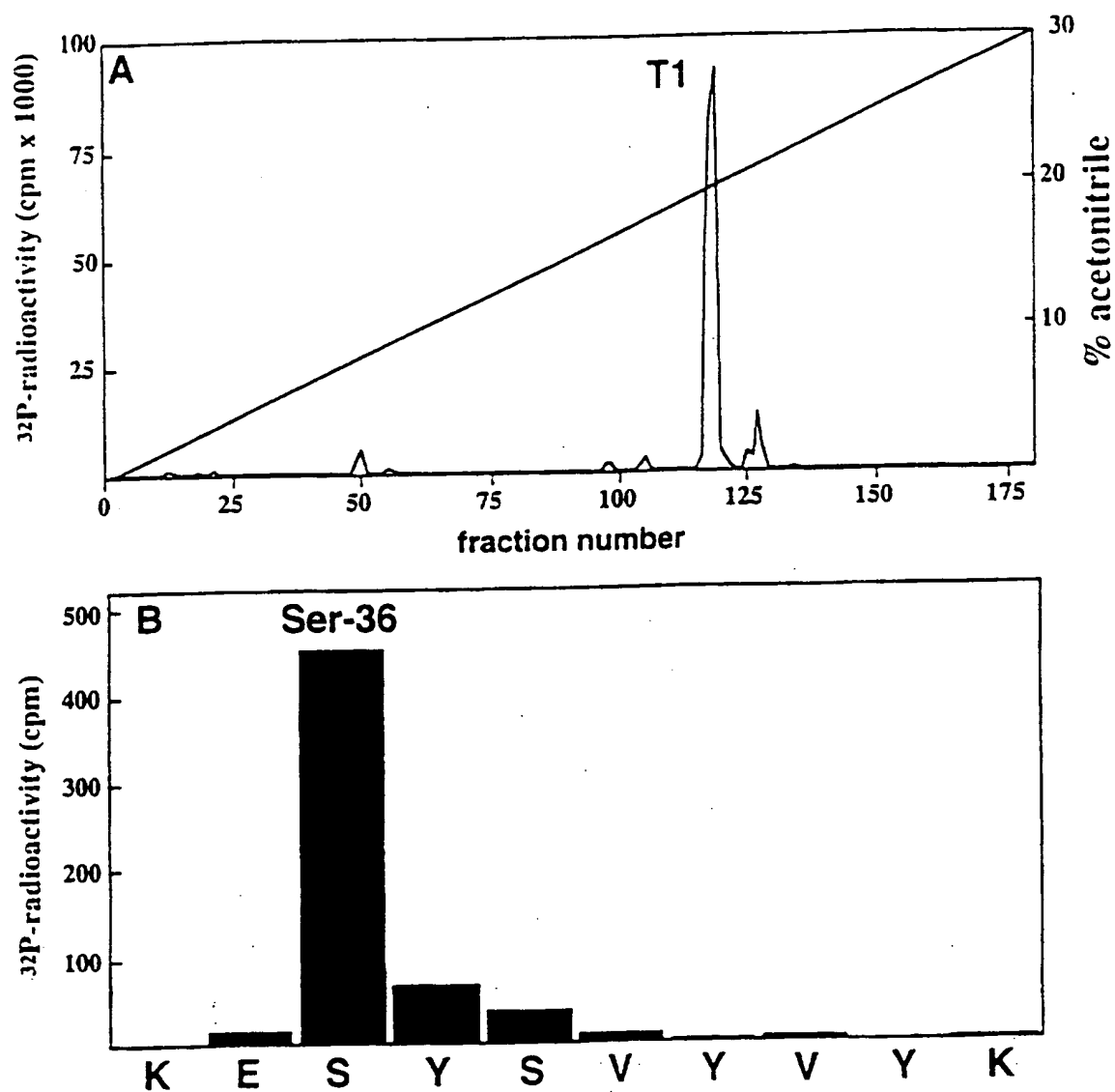
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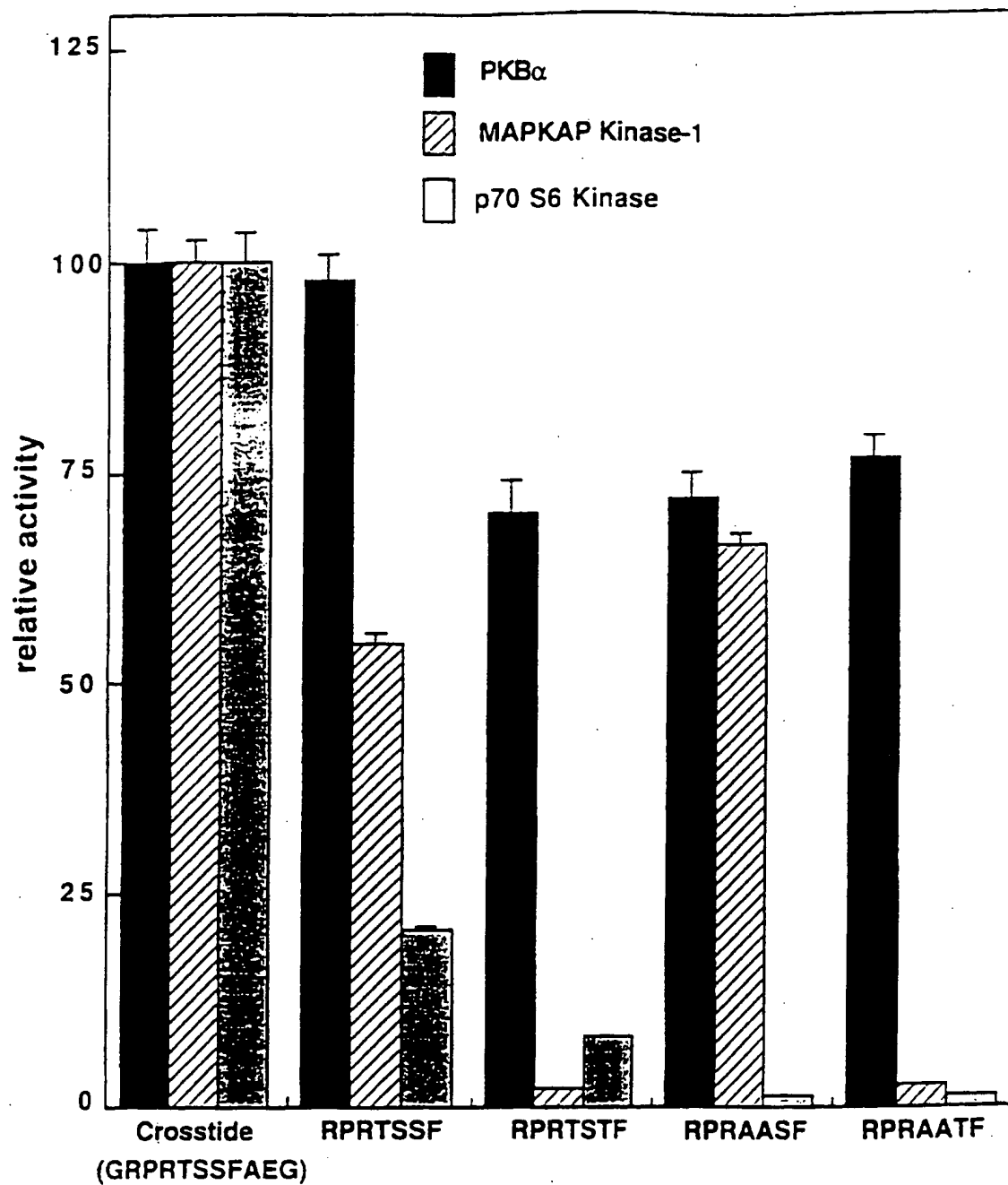
Fig. 18
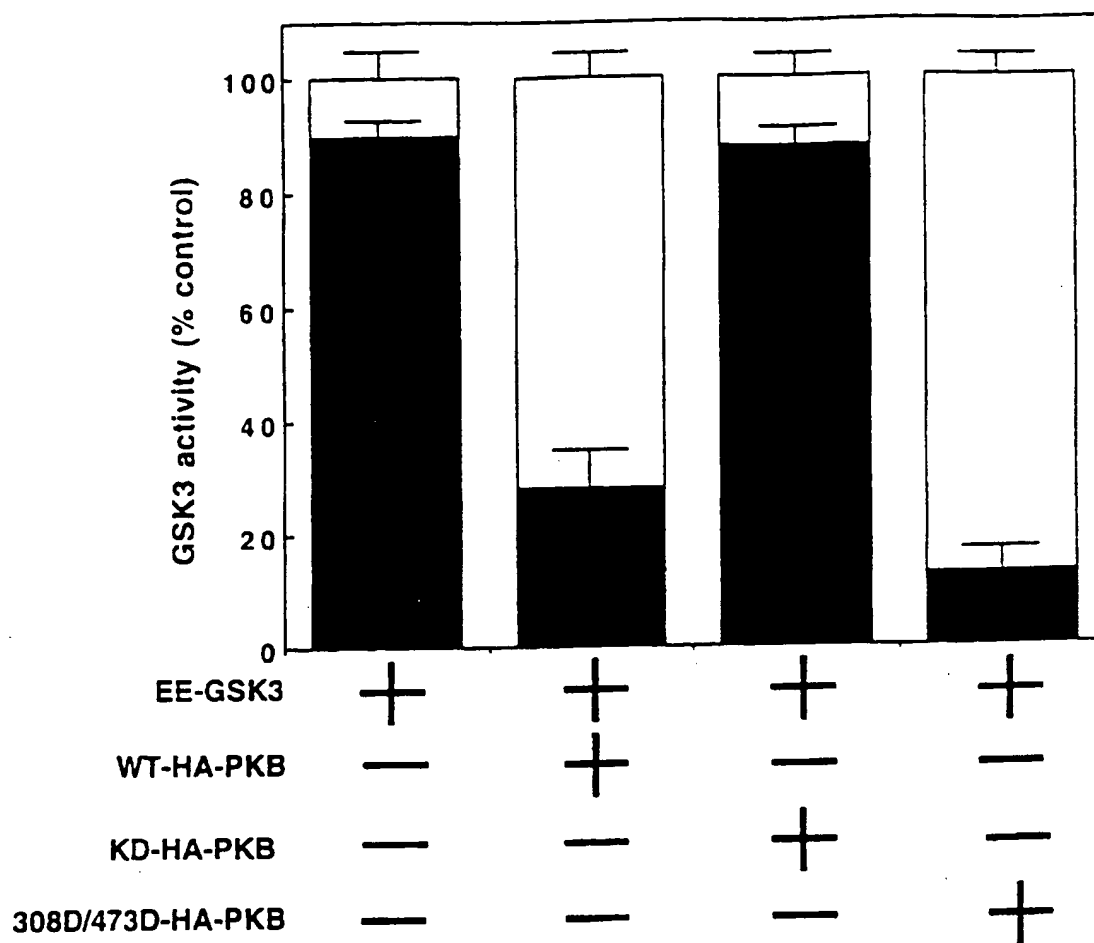
Fig. 19a

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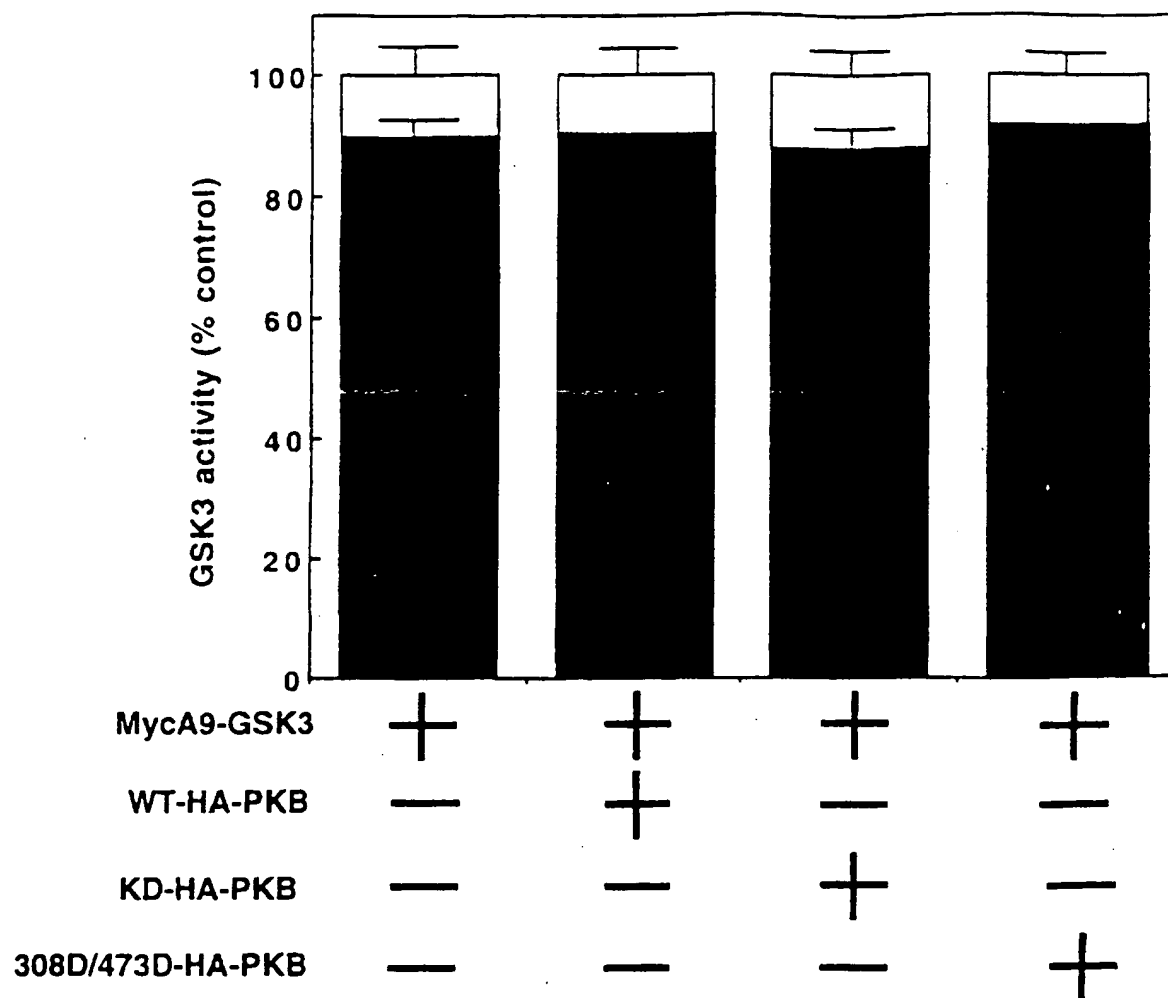
Fig. 19b

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Fig. 20

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Fig. 21